

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
Christensen O'Connor Johnson &
Kindness PLLC
Attn: SHELTON, Dennis K.
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

RECEIVED DOCKETING
FEB 01 2001
CHRISTENSEN O'CONNOR
JOHNSON KINDNESS PLLC

Applicant's or agent's file reference OMER-1-15690	Date of mailing (day/month/year) 29/01/2001
International application No. PCT/US 00/19864	International filing date (day/month/year) 21/07/2000
Applicant OMEROS MEDICAL SYSTEMS, INC.	

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.


☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Joannes Vergoosen
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NOTE FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference OMER-1-15690	FOR FURTHER ACTION <small>see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.</small>	
International application No. PCT/US 00/ 19864	International filing date (day/month/year) 21/07/2000	(Earliest) Priority Date (day/month/year) 21/07/1999
Applicant OMEROS MEDICAL SYSTEMS, INC.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.
☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

INTF NATIONAL SEARCH REPORT

International Application No

P S 00/19864

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K45/06 A61P19/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 26657 A (MEDICAL UNIVERSITY OF SOUTH CAROLINA) 3 June 1999 (1999-06-03) claims 1,34-38 page 57, line 18-23 page 65, line 21-24	1-5,7
P,X	WO 00 23072 A (OMEROS MEDICAL SYSTEMS) 27 April 2000 (2000-04-27) claims 1,3-5,14 page 48, line 12-19 page 53, line 22-30 page 55, line 25-35 page 64, line 20 -page 65, line 14	1-15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

22 January 2001

Date of mailing of the international search report

29/01/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Peeters, J

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-37 relate to a product/compound/method defined by reference to a desirable characteristic or property, namely a first chondroprotective agent and a second chondroprotective agent.

The claims cover all products/compounds/methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products/compounds/methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product/compound/method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for the products/compounds/methods described in the examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATION EARCH REPORT

International application No.
PCT/US 00/19864

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

P/S 00/19864

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9926657	A	03-06-1999	NONE	
WO 0023072	A	27-04-2000	AU 1127700 A	08-05-2000

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SHELTON, Dennis, K.
Christensen, O'Connor, Johnson &
Kindness PLLC
1420 Fifth Avenue, Suite 2800
Seattle, WA 98101
ETATS-UNIS D'AMERIQUE

RECEIVED DOCKETING
JUL 23 2001
CHRISTENSEN O'CONNOR
JOHNSON KINDNESS PLLC

TJB

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

DOCKETED

Date of mailing
(day/month/year) 17.07.2001

Applicant's or agent's file reference
OMER-1-15690

IMPORTANT NOTIFICATION

International application No.
PCT/US00/19864

International filing date (day/month/year)
21/07/2000

Priority date (day/month/year)
21/07/1999

Applicant
OMEROS MEDICAL SYSTEMS, INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Hundt, D

Tel. +49 89 2399-8042





DOCKETED

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference OMER-1-15690	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/19864	International filing date (day/month/year) 21/07/2000	Priority date (day/month/year) 21/07/1999
International Patent Classification (IPC) or national classification and IPC A61K45/06		
Applicant OMEROS MEDICAL SYSTEMS, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 7 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 09/02/2001	Date of completion of this report 17.07.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Peris Antoli, B Telephone No. +49 89 2399 8476



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/19864

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-89 as originally filed

Claims, No.:

1-37 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US00/19864

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 1-37 (partially); 1-27 (industrial applicability).

because:

- ☒ the said international application, or the said claims Nos. 1-27 (industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 1-37 (partially).
2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)

Yes: Claims 1-37 (partially)



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US00/19864

	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-37 (partially)
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	28-37; 1-27 (see separate sheet)
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. As indicated in the international search report, the search has been limited to those parts of the claims relating to the compounds mentioned in the examples.
 - 1.1 According to Rule 66.1(e) PCT, no international preliminary examination will be carried out in respect of the subject matter which is not covered by the search report.
 - 1.2 Hence, for the purpose of this report the claims haven read as if the methods and solutions mentioned therein were restricted to those using or containing the specific combinations of active agents specified in examples 1 to 5 of the present application, namely to a combination of
 - (i) MAP kinase inhibitor, matrix metalloproteinase inhibitor and TGF- β agonist;
 - (ii) MAP kinase inhibitor, nitric oxide synthase inhibitor and interleukin receptor agonist;
 - (iii) MAP kinase inhibitor, nitric oxide synthase inhibitor and TGF- β agonist;
 - (iv) MAP kinase inhibitor and matrix metalloproteinase inhibitor; or
 - (v) BMP receptor agonist, nitric oxide synthase inhibitor and TGF- β agonist.
 - 1.3 For the remaining subject matter of claims 1-37 no opinion with regard to novelty, inventive step and industrial applicability will be established.
2. Claims 1-27 (as far as they refer to the use of the aforementioned combinations (i) to (v)) relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3. Reference is made to the following documents:

D1: WO-A-99 26657

4. As far as claims 1-37 relate to methods or compositions (in form of solutions) using or comprising a combination of active agents selected from

- (i) MAP kinase inhibitor, matrix metalloproteinase inhibitor and TGF- β agonist;
- (ii) MAP kinase inhibitor, nitric oxide synthase inhibitor and interleukin receptor agonist;
- (iii) MAP kinase inhibitor, nitric oxide synthase inhibitor and TGF- β agonist;
- (iv) MAP kinase inhibitor and matrix metalloproteinase inhibitor; or
- (v) BMP receptor agonist, nitric oxide synthase inhibitor and TGF- β agonist,

said claims 1-37 meet the requirements of Art. 33(2) and 33(3) PCT for the reasons set out below.

4.1 D1 is the only prior art in accordance with Rule 64(1) PCT cited in the search report. D1 (see e.g. claims 1-2 and 34-35) discloses the use of certain kind of compounds for suppressing the induction of inducible nitric oxide synthase and the induction of proinflammatory cytokines.

4.2 Compositions comprising a combination as specified in items (i) to (v) of point 4 above, and the possible use of said combinations for inhibiting cartilage degradation are neither disclosed nor suggested in D1.

5. Claims 28-37 satisfy the criterion set forth in Art. 33(4) PCT because their subject matter is susceptible of industrial application.

6. For the assessment of the present claims 1-27 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/US00/19864

patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.



Re Item VI

Certain documents cited

7. WO-A-00/23072 (priority date: 20.10.98; filing date: 20.10.99; publication date: 27.04.00).

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference OMER-1-15690	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/19864	International filing date (day/month/year) 21/07/2000	Priority date (day/month/year) 21/07/1999
International Patent Classification (IPC) or national classification and IPC A61K45/06		
Applicant OMEROS MEDICAL SYSTEMS, INC. et al.		
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 7 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>		
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none">I <input checked="" type="checkbox"/> Basis of the reportII <input type="checkbox"/> PriorityIII <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicabilityIV <input type="checkbox"/> Lack of unity of inventionV <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statementVI <input checked="" type="checkbox"/> Certain documents citedVII <input type="checkbox"/> Certain defects in the international applicationVIII <input type="checkbox"/> Certain observations on the international application		
Date of submission of the demand 09/02/2001	Date of completion of this report 17.07.2001	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Peris Antoli, B Telephone No. +49 89 2399 8476 	

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/19864

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-89 as originally filed

Claims, No.:

1-37 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/19864

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-37 (partially); 1-27 (industrial applicability).

because:

☒ the said international application, or the said claims Nos. 1-27 (industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-37 (partially).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)

Yes: Claims 1-37 (partially)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/19864

	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-37 (partially)
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	28-37; 1-27 (see separate sheet)
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. As indicated in the international search report, the search has been limited to those parts of the claims relating to the compounds mentioned in the examples.
 - 1.1 According to Rule 66.1(e) PCT, no international preliminary examination will be carried out in respect of the subject matter which is not covered by the search report.
 - 1.2 Hence, for the purpose of this report the claims have been read as if the methods and solutions mentioned therein were restricted to those using or containing the specific combinations of active agents specified in examples 1 to 5 of the present application, namely to a combination of
 - (i) MAP kinase inhibitor, matrix metalloproteinase inhibitor and TGF- β agonist;
 - (ii) MAP kinase inhibitor, nitric oxide synthase inhibitor and interleukin receptor agonist;
 - (iii) MAP kinase inhibitor, nitric oxide synthase inhibitor and TGF- β agonist;
 - (iv) MAP kinase inhibitor and matrix metalloproteinase inhibitor; or
 - (v) BMP receptor agonist, nitric oxide synthase inhibitor and TGF- β agonist.
 - 1.3 For the remaining subject matter of claims 1-37 no opinion with regard to novelty, inventive step and industrial applicability will be established.
2. Claims 1-27 (as far as they refer to the use of the aforementioned combinations (i) to (v)) relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3. Reference is made to the following documents:

D1: WO-A-99 26657

4. As far as claims 1-37 relate to methods or compositions (in form of solutions) using or comprising a combination of active agents selected from
- (i) MAP kinase inhibitor, matrix metalloproteinase inhibitor and TGF- β agonist;
 - (ii) MAP kinase inhibitor, nitric oxide synthase inhibitor and interleukin receptor agonist;
 - (iii) MAP kinase inhibitor, nitric oxide synthase inhibitor and TGF- β agonist;
 - (iv) MAP kinase inhibitor and matrix metalloproteinase inhibitor; or
 - (v) BMP receptor agonist, nitric oxide synthase inhibitor and TGF- β agonist,

said claims 1-37 meet the requirements of Art. 33(2) and 33(3) PCT for the reasons set out below.

- 4.1 D1 is the only prior art in accordance with Rule 64(1) PCT cited in the search report. D1 (see e.g. claims 1-2 and 34-35) discloses the use of certain kind of compounds for suppressing the induction of inducible nitric oxide synthase and the induction of proinflammatory cytokines.
- 4.2 Compositions comprising a combination as specified in items (i) to (v) of point 4 above, and the possible use of said combinations for inhibiting cartilage degradation are neither disclosed nor suggested in D1.
5. Claims 28-37 satisfy the criterion set forth in Art. 33(4) PCT because their subject matter is susceptible of industrial application.
6. For the assessment of the present claims 1-27 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/US00/19864

patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI

Certain documents cited

7. WO-A-00/23072 (priority date: 20.10.98; filing date: 20.10.99; publication date: 27.04.00).

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(54) Title: SOLUTIONS AND METHODS FOR INHIBITION OF PAIN, INFLAMMATION AND CARTILAGE DEGRADATION

(57) Abstract: Methods and solutions for inhibiting a variety of pain and inflammation processes at wounds from general surgical procedures including arthroscopic procedures, and for inhibiting cartilage degradation are disclosed. The solutions preferably include multiple pain and inflammation inhibitory at dilute concentration in a physiologic carrier, such as saline or lactated Ringer's solution. The solution may be applied by continuous irrigation of a wound during a surgical procedure for preemptive inhibition of pain and while avoiding undesirable side effects associated with oral, intramuscular, subcutaneous or intravenous application of larger doses of the agents. Alternatively, for combinations of cartilage degradation inhibitors, the solutions may be injected directly into the joint.

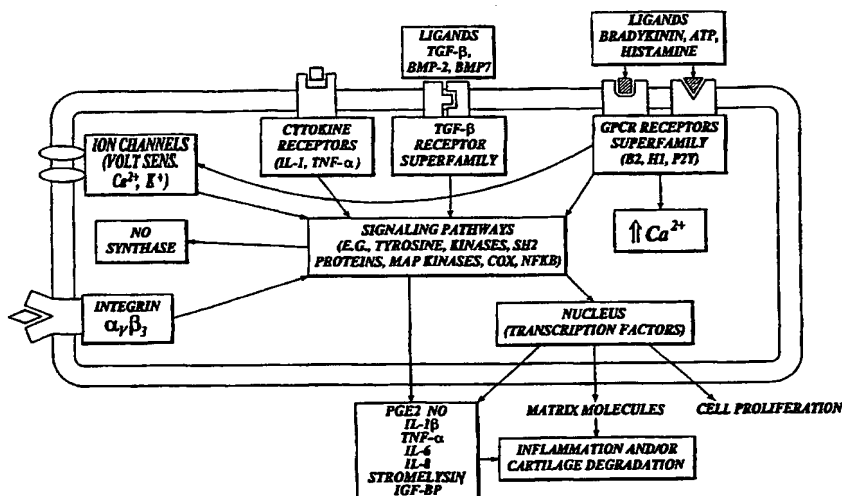
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- (74) Agent: **SHELTON, Dennis, K.**; Christensen O'Connor Johnson & Kindness PLLC, Suite 2800, 1420 Fifth Avenue, Seattle, WA 98101 (US).
- (81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.**
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(54) Title: **SOLUTIONS AND METHODS FOR INHIBITION OF PAIN, INFLAMMATION AND CARTILAGE DEGRADATION****CHONDROCYTE CELL MOLECULAR TARGETS & SIGNALING INFORMATION**

(57) Abstract: Methods and solutions for inhibiting a variety of pain and inflammation processes at wounds from general surgical procedures including arthroscopic procedures, and for inhibiting cartilage degradation are disclosed. The solutions preferably include multiple pain and inflammation inhibitory at dilute concentration in a physiologic carrier, such as saline or lactated Ringer's solution. The solution may be applied by continuous irrigation of a wound during a surgical procedure for preemptive inhibition of pain and while avoiding undesirable side effects associated with oral, intramuscular, subcutaneous or intravenous application of larger doses of the agents. Alternatively, for combinations of cartilage degradation inhibitors, the solutions may be injected directly into the joint.

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SOLUTIONS AND METHODS FOR INHIBITION OF PAIN, INFLAMMATION AND CARTILAGE DEGRADATION

I. Field of the Invention

5 The present invention relates to therapeutic solutions and methods, and particularly for anti-inflammatory, anti-pain, and anti-cartilage degradation solutions and methods.

II. Background of the Invention

10 Arthroscopy is a surgical procedure in which a camera, attached to a remote light source and video monitor, is inserted into an anatomic joint (e.g., knee, shoulder, etc.) through a small portal incision in the overlying skin and joint capsule. Through similar portal incisions, surgical instruments may be placed in the joint, their use guided by arthroscopic visualization. As arthroscopists' skills have improved, an increasing number of operative procedures, once performed by "open" surgical technique, now can be accomplished arthroscopically. Such procedures include, for
15 example, partial meniscectomies and ligament reconstructions in the knee, shoulder acromioplasties and rotator cuff debridements and elbow synovectomies. As a result of widening surgical indications and the development of small diameter arthroscopes, wrist and ankle arthroscopies also have become routine.

20 Throughout each arthroscopy, physiologic irrigation fluid (e.g., normal saline or lactated Ringer's) is flushed continuously through the joint, distending the joint capsule and removing operative debris, thereby providing clearer intra-articular visualization. U.S. Patent 4,504,493 to Marshall discloses an isomolar solution of glycerol in water for a non-conductive and optically clear irrigation solution for

arthroscopy. Conventional physiologic irrigation fluids do not provide analgesic, anti-inflammatory and anti-cartilage degradation effects.

Alleviating pain and suffering in postoperative patients is an area of special focus in clinical medicine, especially with the growing number of outpatient operations performed each year. The most widely used systemic agents, cyclooxygenase inhibitors (e.g., ibuprofen) and opioids (e.g., morphine, fentanyl), have significant side effects including gastrointestinal irritation/bleeding and respiratory depression. The high incidence of nausea and vomiting related to opioids is especially problematic in the postoperative period. Therapeutic agents aimed at treating postoperative pain while avoiding detrimental side effects are not easily developed because the molecular targets for these agents are distributed widely throughout the body and mediate diverse physiological actions. Despite the significant clinical need to inhibit pain and inflammation, as well as cartilage degradation, methods for the delivery of inhibitors of pain, inflammation, and cartilage degradation at effective dosages while minimizing adverse systemic side effects have not been developed. As an example, conventional (i.e., intravenous, oral, subcutaneous or intramuscular) methods of administration of opiates in therapeutic doses frequently is associated with significant adverse side effects, including severe respiratory depression, changes in mood, mental clouding, profound nausea and vomiting.

Prior studies have demonstrated the ability of endogenous agents, such as serotonin (5-hydroxytryptamine, sometimes referred to herein as "5-HT"), bradykinin and histamine, to produce pain and inflammation. Sicuteri, F., et. al., *Serotonin-Bradykinin Potentiation in the Pain Receptors in Man*, Life Sci. 4, pp. 309-316 (1965); Rosenthal, S.R., *Histamine as the Chemical Mediator for Cutaneous Pain*, J. Invest. Dermat. 69, pp. 98-105 (1977); Richardson, B.P., et. al., *Identification of Serotonin M-Receptor Subtypes and their Specific Blockade by a New Class of Drugs*, Nature 316, pp. 126-131 (1985); Whalley, E.T., et. al., *The Effect of Kinin Agonists and Antagonists*, Naunyn-Schmiedeb Arch. Pharmacol. 36, pp. 652-57 (1987); Lang, E., et. al., *Chemo-Sensitivity of Fine Afferents from Rat Skin In Vitro*, J. Neurophysiol. 63, pp. 887-901 (1990).

For example, 5-HT applied to a human blister base (denuded skin) has been demonstrated to cause pain that can be inhibited by 5-HT₃ receptor antagonists. Richardson et al., (1985). Similarly, peripherally-applied bradykinin produces pain which can be blocked by bradykinin receptor antagonists. Sicuteri et al., 1965; Whalley et al., 1987; Dray, A., et. al., *Bradykinin and Inflammatory Pain*, Trends

Neurosci. 16, pp. 99-104 (1993). Peripherally-applied histamine produces vasodilation, itching and pain which can be inhibited by histamine receptor antagonists. Rosenthal, 1977; Douglas, W.W., "Histamine and 5-Hydroxytryptamine (Serotonin) and their Antagonists", in Goodman, L.S., et. al., ed., *The Pharmacological Basis of Therapeutics*, MacMillan Publishing Company, New York, pp. 605-638 (1985); Rumore, M.M., et. al., *Analgesic Effects of Antihistaminics*, Life Sci 36, pp. 403-416 (1985). Combinations of these three agonists (5-HT, bradykinin and histamine) applied together have been demonstrated to display a synergistic pain-causing effect, producing a long-lasting and intense pain signal. Sicuteri et al., 1965; Richardson et al., 1985; Kessler, W., et. al., "Excitation of Cutaneous Afferent Nerve Endings In Vitro by a Combination of Inflammatory Mediators and Conditioning Effect of Substance P," *Exp. Brain Res.* 91: 467-476 (1992).

In the body, 5-HT is located in platelets and in central neurons, histamine is found in mast cells, and bradykinin is produced from a larger precursor molecule during tissue trauma, pH changes and temperature changes. Because 5-HT can be released in large amounts from platelets at sites of tissue injury, producing plasma levels 20-fold greater than resting levels (Ashton, J.H., et. al., "Serotonin as a Mediator of Cyclic Flow Variations in Stenosed Canine Coronary Arteries," *Circulation* 73: 572-578 (1986)), it is possible that endogenous 5-HT plays a role in producing postoperative pain, hyperalgesia and inflammation. In fact, activation of platelets has been shown to result in excitation of peripheral nociceptors *in vitro*. Ringkamp, M., et. al., "Activated Human Platelets in Plasma Excite Nociceptors in Rat Skin, *In Vitro*," *Neurosci. Lett.* 170: 103-106 (1994). Similarly, histamine and bradykinin also are released into tissues during trauma. Kimura, E., et. al., "Changes in Bradykinin Level in Coronary Sinus Blood After the Experimental Occlusion of a Coronary Artery," *Am Heart J.* 85: 635-647 (1973); Douglas, 1985; Dray et. al. (1993).

In addition, prostaglandins also are known to cause pain and inflammation. Cyclooxygenase inhibitors, e.g., ibuprofen, are commonly used in non-surgical and post-operative settings to block the production of prostaglandins, thereby reducing prostaglandin-mediated pain and inflammation. Flower, R.J., et. al., *Analgesic-Antipyretics and Anti-Inflammatory Agents; Drugs Employed in the Treatment of Gout*, in Goodman, L.S., et. al., ed., *The Pharmacological Basis of Therapeutics*, MacMillan Publishing Company, New York, pp. 674-715 (1985). Cyclooxygenase inhibitors are associated with some adverse systemic side effects when applied

conventionally. For example, indomethacin or ketorolac have well recognized gastrointestinal and renal adverse side effects.

As discussed, 5-HT, histamine, bradykinin and prostaglandins cause pain and inflammation. The various receptors through which these agents mediate their effects on peripheral tissues have been known and/or debated for the past two decades. Most studies have been performed in rats or other animal models. However, there are differences in pharmacology and receptor sequences between human and animal species.

Furthermore, antagonists of these mediators currently are not used for postoperative pain treatment. A class of drugs, termed 5-HT and norepinephrine uptake antagonists, which includes amitriptyline, has been used orally with moderate success for chronic pain conditions. However, the mechanisms of chronic versus acute pain states are thought to be considerably different. In fact, two studies in the acute pain setting using amitriptyline perioperatively have shown no pain-relieving effect of amitriptyline. Levine, J.D. et. al., "Desipramine Enhances Opiate Postoperative Analgesia, *Pain* 27: 45-49 (1986); Kerrick, J.M. et. al., "Low-Dose Amitriptyline as an Adjunct to Opioids for Postoperative Orthopedic Pain: a Placebo-Controlled Trial Period," *Pain* 52: 325-30 (1993). In both studies the drug was given orally. The second study noted that oral amitriptyline actually produced a lower overall sense of well being in postoperative patients, which may be due to the drug's affinity for multiple amine receptors in the brain.

Amitriptyline, in addition to blocking the uptake of 5-HT and norepinephrine, is a potent 5-HT receptor antagonist. Therefore, the lack of efficacy in reducing postoperative pain in the previously mentioned studies would appear to conflict with the proposal of a role for endogenous 5-HT in acute pain. There are a number of reasons for the lack of acute pain relief found with amitriptyline in these two studies. (1) The first study (Levine et al., 1986) used amitriptyline preoperatively for one week up until the night prior to surgery whereas the second study (Kerrick et al., 1993) only used amitriptyline postoperatively. Therefore, the level of amitriptyline that was present in the operative site tissues during the actual tissue injury phase, and the time at which 5-HT is purported to be released, is unknown. (2) Amitriptyline is known to be extensively metabolized by the liver. With oral administration, the concentration of amitriptyline in the operative site tissues may not have been sufficiently high for a long enough time period to inhibit the activity of postoperatively released 5-HT in the second study. (3) Since multiple inflammatory mediators exist, and studies have demonstrated synergism between the inflammatory

mediators, blocking only one agent (5-HT) may not sufficiently inhibit the inflammatory response to tissue injury.

There have been a few studies demonstrating the ability of extremely high concentrations (1% - 3% solutions -- i.e., 10 - 30 mg per milliliter) of histamine₁ (H₁) receptor antagonists to act as local anesthetics for surgical procedures. This anesthetic effect is not believed to be mediated via H₁ receptors but, rather, due to a non-specific interaction with neuronal membrane sodium channels (similar to the action of lidocaine). Given the side effects (e.g., sedation) associated with these high "anesthetic" concentrations of histamine receptor antagonists, local administration of histamine receptor antagonists currently is not used in the perioperative setting.

III. Summary of the Invention

The present invention provides a solution constituting a mixture of multiple agents in low concentrations directed at inhibiting locally the mediators of pain, inflammation, and cartilage degradation in a physiologic electrolyte carrier fluid. The invention also provides a method for perioperative delivery of the irrigation solution containing these agents directly to a surgical site, where it works locally at the receptor and enzyme levels to preemptively limit pain, inflammation, and cartilage degradation at the site. Due to the local perioperative delivery method of the present invention, a desired therapeutic effect can be achieved with lower doses of agents than are necessary when employing other methods of delivery (i.e., intravenous, intramuscular, subcutaneous and oral). The anti-pain and/or anti-inflammation agents and/or anti-cartilage degradation agents in the solution include agents selected from the following classes of receptor antagonists and agonists and enzyme activators and inhibitors, each class acting through a differing molecular mechanism of action for pain and/or inflammation inhibition and/or cartilage degradation. Representative agents for the inhibition of pain and/or inflammation include, for example: (1) serotonin receptor antagonists; (2) serotonin receptor agonists; (3) histamine receptor antagonists; (4) bradykinin receptor antagonists; (5) kallikrein inhibitors; (6) tachykinin receptor antagonists, including neurokinin₁ and neurokinin₂ receptor subtype antagonists; (7) calcitonin gene-related peptide (CGRP) receptor antagonists; (8) interleukin receptor antagonists; (9) inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, including (a) phospholipase inhibitors, including PLA₂ isoform inhibitors and PLC isoform inhibitors, (b) cyclooxygenase inhibitors, and (c) lipoxygenase inhibitors; (10) prostanoid receptor antagonists including eicosanoid EP-1 and EP-4 receptor subtype antagonists and thromboxane receptor subtype antagonists; (11) leukotriene receptor antagonists including

leukotriene B₄ receptor subtype antagonists and leukotriene D₄ receptor subtype antagonists; (12) opioid receptor agonists, including μ -opioid, δ -opioid, and κ -opioid receptor subtype agonists; (13) purinoceptor antagonists including P_{2X} receptor antagonists and P_{2Y} receptor antagonists; and (14) calcium channel antagonists.

5 Each of the above agents functions either as an anti-inflammatory agent and/or as an anti-nociceptive, i.e., anti-pain or analgesic, agent. The selection of agents from these classes of compounds is tailored for the particular application. Representative agents for the inhibition of cartilage degradation include, for example:

10 (1) antagonists of receptors for the interleukin-1 family of proteins, including, for example, IL-1 β , IL-17 and IL-18; (2) antagonists of the tumor necrosis factor (TNF) receptor family, including, for example, TNF-R1; (3) agonists for interleukin 4, 10 and 13 receptors; (4) agonists for the TGF- β receptor superfamily, including, for example, BMP-2, BMP-4 and BMP-7; (5) inhibitors of COX-2; (6) inhibitors of the MAP kinase family, including, for example, p38 MAP kinase; (7) inhibitors of the

15 matrix metalloproteinases (MMP) family of proteins, including, for example, MMP-3 and MMP-9; (8) inhibitors of the NF- κ B family of proteins, including, for example, the p50/p65 dimer complex with I κ B; (9) inhibitors of the nitric oxide synthase (NOS) family, including, for example, iNOS; (10) agonists and antagonists of integrin receptors, including, for example, agonists of $\alpha_v\beta_3$ integrin; (11) inhibitors

20 of the protein kinase C (PKC) family; (12) inhibitors of the protein tyrosine kinase family, including, for example, the src subfamily; (13) modulators of protein tyrosine phosphatases; and (14) inhibitors of protein src homology 2 (SH2) domains.

In yet other aspects of the invention, methods and solutions are provided for reducing or preventing destruction of articular cartilage in a joint, by administering

25 directly to the joint of a patient a composition which includes one or more metabolically active chondroprotective agents together with one or more agents for the inhibition of pain, inflammation, or the like, as previously described, or alternatively a combination of two or more metabolically active chondroprotective agents in a pharmaceutically effective carrier for intra-articular delivery.

30 Metabolically active agents include, but are not limited to, compounds that act directly or indirectly to modulate or alter the biological, biochemical or biophysical state of a cell, including agents that alter the electrical potential of the plasma membrane, the ligand binding or enzymatic activity of cellular receptors, intracellular or extracellularly located enzymes, protein-protein interactions, RNA-protein

35 interactions, or DNA-protein interactions. In one aspect of the present invention pharmaceutical compositions of metabolically active chondroprotective agents are

provided that are based upon a combination of at least two agents that act simultaneously on distinct molecular targets. In a representative embodiment, at least one agent is a cytokine or growth factor receptor agonist that directly provides anti-inflammatory activity and/or promotes cartilage anabolic processes and at least a second agent is a receptor antagonist or enzyme inhibitor that acts to inhibit pro-inflammatory and/or cartilage catabolic processes. Anti-inflammatory/anabolic cytokines, which act functionally to suppress the role of pro-inflammatory cytokines in the joint, promote cartilage matrix synthesis and inhibit matrix degradation. These receptor agonists include, for example, specific anti-inflammatory and anabolic cytokines, such as the interleukin (IL) agonists (e.g., IL-4, IL-10 and IL-13) and specific members of the transforming growth factor- β superfamily (e.g., TGF β and BMP-7), insulin-like growth factors (e.g., IGF-1) and fibroblast growth factors (e.g., bFGF). At least a second agent is drawn from a class of receptor antagonists or enzyme inhibitors that acts to inhibit and reduce the activity or the expression of a pro-inflammatory molecular target (e.g., the IL-1 receptor antagonists, TNF- α receptor antagonists, cyclooxygenase-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase (NOS) inhibitors, and nuclear factor kappaB (NF κ B) inhibitors). The multiple agent combination of anabolic agents and inhibitors of catabolism can be delivered locally by intra-articular injection or via infusion, including administration periprocedurally (ie., pre-operatively and/or intra-operatively and/or post-operatively) during surgical arthroscopic procedures.

Articular cartilage is a specialized extracellular matrix that is produced and maintained by metabolically active articular chondrocytes. The maintenance of a normal, healthy extracellular matrix reflects a dynamic balance between the rate of biosynthesis and incorporation of matrix components, and the rate of their degradation and subsequent loss from the cartilage into the synovial fluid. While the regulatory mechanisms that underlie the matrix homeostasis are not well understood, they are clearly altered in inflammatory joint diseases and in response to joint trauma such that the rate of matrix breakdown exceeds the rate of new synthesis of matrix components. Matrix homeostasis is generally regarded to represent a dynamic balance between the effects of catabolic cytokines and anabolic cytokines (including growth factors). The optimal combination of therapeutic agents useful for cartilage protection shifts the dynamic matrix equilibrium through accelerating the synthetic rate and simultaneously inhibiting the rate of breakdown, thus maximizing anabolic processes and promoting repair.

Catabolic cytokines, such as IL-1 β and TNF- α act at specific receptors on chondrocytes to induce production of MMPs that induce matrix degradation while the degradation is inhibited by anabolic cytokines such as TGF- β , BMP-2 and IGF-1. Hence, a therapeutic approach that is based only upon inhibiting catabolic processes (such as a combination of an MMP inhibitor and an IL-1 antagonist) is not optimal for cartilage repair since anabolic agents are needed to induce or accelerate biosynthesis and assembly of components for matrix production. Secondly, the multiplicity of catabolic cytokines (IL-1, TNF, IL-17, IL-18, LIF) that contribute to cartilage matrix destruction indicate it will not be practical to block all the catabolic cytokine activity. Conversely, an approach that relies only upon use of anabolic agents, such as IGF-1, BMP-2 or BMP-7, is not optimal since it does not address the counter-regulatory role of the catabolic cytokines. TGF- β , BMP-2 and IGF-1 also act at specific receptors to induce chondrocytes to produce matrix components, which is inhibited by IL-1 β , TNF- α , IL-17 and LIF. Therefore, the optimal therapeutic combination for chondroprotection is composed of at least one anabolic agent and one inhibitor of cartilage catabolism.

The present invention also provides a method for manufacturing a medicament compounded as a dilute irrigation solution for use in continuously irrigating an operative site, typically at the site of a joint of a patient, during an arthroscopic operative procedure. The method entails dissolving in a physiologic electrolyte carrier fluid at least one anti-cartilage degradation agent and preferably one or more anti-pain/anti-inflammatory agents, and for some applications anti-cartilage degradation agents, each agent included at a concentration of preferably no more than about 100,000 nanomolar, more preferably no more than about 25,000 nanomolar, and most preferably no more than about 10,000 nanomolar.

The method of the present invention provides for the delivery of a dilute combination of multiple receptor antagonists and agonists and enzyme inhibitors and activators directly to a wound or operative site, during therapeutic or diagnostic procedures for the inhibition of pain, inflammation and cartilage degradation. Since the active ingredients in the solution are being locally applied directly to the operative tissues in a continuous fashion, the drugs may be used efficaciously at extremely low doses relative to those doses required for therapeutic effect when the same drugs are delivered orally, intramuscularly, subcutaneously or intravenously. As used herein, the term "local" encompasses application of a drug in and around a wound or other operative site, and excludes oral, subcutaneous, intravenous and intramuscular administration. The term "continuous" as used herein encompasses uninterrupted

application, repeated application at frequent intervals, and applications which are uninterrupted except for brief cessations such as to permit the introduction of other drugs or agents or procedural equipment, such that a substantially constant predetermined concentration is maintained locally at the wound or operative site.

5 The advantages of low dose applications of agents are three-fold. The most important is the absence of systemic side effects which often limit the usefulness of these agents. Additionally, the agents selected for particular applications in the solutions of the present invention are highly specific with regard to the mediators and mediation targets on which they work. This specificity is maintained by the low
10 dosages utilized. Finally, the cost of these active agents per operative procedure is low.

 The advantages of local administration of the agents via irrigation or other fluid application are the following: (1) local administration guarantees a known concentration at the target site, regardless of interpatient variability in metabolism,
15 blood flow, etc.; (2) because of the direct mode of delivery, a therapeutic concentration is obtained instantaneously and, thus, improved dosage control is provided; and (3) local administration of the active agents directly to a wound or operative site also substantially reduces degradation of the agents through systemic processes, e.g., first- and second-pass metabolism, that would otherwise occur if the
20 agents were given orally, intravenously, subcutaneously or intramuscularly. This is particularly true for those active agents that are peptides, which are metabolized rapidly. Thus, local administration permits the use of compounds or agents which otherwise could not be employed therapeutically. For example, some agents in the following classes are peptidic: bradykinin receptor antagonists; tachykinin receptor
25 antagonists; opioid receptor agonists; CGRP receptor antagonists; and interleukin receptor antagonists, TNF-receptor antagonists; TGF- β receptor agonists; BMP-2 and BMP-7 receptor agonists; IL4, IL10 and IL-13 receptor agonists; and integrin receptor agonists and antagonists. Local, continuous delivery to the wound or operative site minimizes drug degradation or metabolism while also providing for the
30 continuous replacement of that portion of the agent that may be degraded, to ensure that a local therapeutic concentration, sufficient to maintain receptor occupancy or enzymatic saturation, is maintained throughout the duration of the operative procedure.

 Local administration of the solution perioperatively throughout a surgical
35 procedure in accordance with the present invention produces a preemptive analgesic, anti-inflammatory and cartilage protective effect. As used herein, the term

“perioperative” encompasses application intraprocedurally, pre- and intraprocedurally, intra- and postprocedurally, and pre-, intra- and postprocedurally. To maximize the preemptive anti-inflammatory, analgesic (for certain applications) and cartilage protective (for certain applications) effects, the solutions of the present invention are most preferably applied pre-, intra- and postoperatively. By occupying the target receptors or inactivating or activating targeted enzymes prior to the initiation of significant operative trauma locally, the agents of the present solution modulate specific pathways to preemptively inhibit the targeted pathologic process. If inflammatory mediators and processes are preemptively inhibited in accordance with the present invention before they can exert tissue damage, the benefit is more substantial than if given after the damage has been initiated.

Inhibiting more than one pain, inflammatory or cartilage degradation mediator by application of the multiple agent solution of the present invention has been shown to dramatically reduce the degree of inflammation and pain, and theoretically should provide a cartilage protective effect. The irrigation solutions of the present invention include combinations of drugs, each solution acting on multiple receptors or enzymes. The drug agents are thus simultaneously effective against a combination of pathologic processes, including pain and inflammation, and loss of cartilage homeostasis. The action of these agents is considered to be synergistic, in that the multiple receptor antagonists and inhibitory agonists of the present invention provide a disproportionately increased efficacy in combination relative to the efficacy of the individual agents. The synergistic action of several of the agents of the present invention are discussed, by way of example, below in the detailed descriptions of those agents.

Used perioperatively, the solution should result in a clinically significant decrease in operative site pain and inflammation, and of cartilage degradation, relative to currently-used irrigation fluids, thereby decreasing the patient's postoperative analgesic (i.e., opiate) requirement and, where appropriate, allowing earlier patient mobilization of the operative site. No extra effort on the part of the surgeon and operating room personnel is required to use the present solution relative to conventional irrigation fluids. For optimum chondroprotection, the solutions of the invention are administered directly to a joint prior to, during and/or after a surgical procedure.

IV. Brief Description of the Drawings

The present invention will now be described in greater detail, by way of example, with reference to the accompanying drawings in which:

FIGURE 1 is a schematic overview of a chondrocyte cell showing molecular targets and flow of signaling information leading to the production of mediators of inflammation and shifts in cartilage metabolism. The integration of extrinsic signals through several families of cell surface receptors, including cytokine receptor such as the interleukin-1 (IL-1) receptor family and the tumor necrosis factor (TNF) receptor family, the TGF- β receptor superfamily and integrins is shown to converge on common intracellular signaling pathways that include major groups of protein molecules that are therapeutic targets of drugs included in the solutions of the present invention (MAP kinases, PKC, tyrosine kinases, SH2 proteins, COX, PLA2 and NF-6B). Activation of these signaling pathways controls chondrocyte expression of a number of inducible gene products, including IL-1, TNF- α , IL-6, IL-8 and Stromelysin (MMP-3), and other mediators (nitric oxide (NO) and PGE2) which may lead to inflammation and/or cartilage degradation, or synthesis of matrix molecules and chondrocyte proliferation;

FIGURE 2 provides a a schematic overview of a synoviocyte cell showing molecular targets and flow of signaling information leading to the production of mediators of inflammation and shifts in cartilage metabolism. The integration of extrinsic signals through several families of cell surface receptors, including cytokine receptors which include the interleukin-1 (IL-1) receptor family and the tumor necrosis factor (TNF) receptor family, the G-protein coupled receptors which include bradykinin, histamine and serotonin subtypes, and integrins is shown to converge on common intracellular signaling pathways that include major groups of protein molecules that are therapeutic targets of drugs included in the solutions of the present invention (MAP kinases, PKC, tyrosine kinases, SH2 proteins, COX, PLA2 and NF-6B). Activation of these signaling pathways controls synoviocyte expression of a number of inducible gene products, including IL-1, TNF- α , IL-6, IL-8 and Stromelysin (MMP-3), which may lead to inflammation and/or cartilage degradation;

FIGURE 3 is a a diagram of common signaling pathways in both chondrocyte and synoviocyte cells, including key signaling proteins responsible for "crosstalk" between GPCR activated receptor pathways and pro-inflammatory cytokine pathways that lead to inflammation and or cartilage degradation;

FIGURE 4 is a a diagram of of common signaling pathways in both chondrocyte and synoviocyte cells , including key signaling proteins responsible for "crosstalk" between GPCR activated receptor pathways and pro-inflammatory cytokine pathways. Specific moelcular sites of action for some drugs in a preferred chondroprotective solution of the present invention are identified;

FIGURE 5 is a diagram of molecular targets present on either chondrocytes or synoviocytes that promote an anabolic response of cartilage. Specific sites of action of some drugs in the preferred chondroprotective solution of the present are identified;

5 FIGURE 6 is a diagram of molecular targets present on either chondrocytes or synoviocytes that promote a catabolic response in cartilage. Specific sites of action of some drugs in the preferred chondroprotective solution of the present invention are identified;

10 FIGURE 7 is a graphical representation of the production of prostaglandin E2 in synovial cultures by G-protein regulatory agonists following overnight priming with interleukin-1 (IL-1, 10U/ml). The cultures were stimulated for the indicated times with histamine (100 μ M, open bars), or bradykinin (1 μ M, closed bars), and the prostaglandin E2 released to the culture supernatant was determined as described in Example 6. The values shown are the mean \pm the standard deviation from a
15 representative experiment, and are corrected for basal prostaglandin E2 production by unstimulated cultures;

FIGURE 8 is a graphical representation of the inhibition of prostaglandin E2 production in synovial cultures by ketoprofen. The cultures were primed overnight with IL-1 (10U/ml) in the presence (shown as "■") or absence (shown as "△" or
20 "▽") of the indicated concentrations of ketoprofen. After one day, prostaglandin E2 was measured in the supernatants of cultures treated overnight with ketoprofen, and the remaining cultures were washed, incubated for 10 minutes with the indicated concentrations of ketoprofen, and then prostaglandin E2 production was measured in response to a subsequent 3 minute challenge with histamine (100 μ M, ▽) or
25 bradykinin (1 μ M, △) in the continuing presence of the indicated amounts of ketoprofen. The data shown are normalized to the maximum response obtained for each agonist, respectively, and represent the mean \pm the standard deviation from three experiments performed on different cell lines; and

FIGURE 9 is a graphical representation of the effect of ketoprofen on IL-6
30 production by synovial cultures at 16 hours in the presence of the indicated concentrations of IL-1 plus the added G-protein coupled receptor ligands. Cultures were incubated for 16 hours with IL-1 at the indicated concentration (0.3, 1.0 and 3.0 pg/ml) in the absence and presence of 0.75 μ M ketoprofen in experimental growth medium with one of the following additional receptor ligands: 1) isoproterenol (ISO)
35 at 1.0 μ M to activate the camp pathway, or 2) histamine (HIS) at 100 μ M to activate the IP3/calcium pathway. Culture supernatants were collected and replaced with

fresh media aliquots containing the same agonist additions at 8 hour intervals. Following treatment, the supernatant medium corresponding to the treatment interval from 8 to 16 hours was collected and analyzed for IL-6.

V. Detailed Description of the Preferred Embodiment

5 The irrigation and injectable solutions of the present invention are dilute solutions of one or more chondroprotective agents and, optionally, one or more pain and/or inflammation inhibitory agents in a physiologic carrier. The carrier is a liquid solution, which as used herein is intended to encompass biocompatible solvents, suspensions, polymerizable and non-polymerizable gels, pastes and salves, as well as
10 components of sustained release delivery systems, such as microparticles, microspheres or nanoparticles composed of proteins, liposomes, carbohydrates, synthetic organic compounds, or inorganic compounds. Preferably the carrier is an aqueous solution that may include physiologic electrolytes, such as normal saline or lactated Ringer's solution.

15 The anti-inflammation and/or anti-pain agents are selected from the group consisting of: (1) serotonin receptor antagonists; (2) serotonin receptor agonists; (3) histamine receptor antagonists; (4) bradykinin receptor antagonists; (5) kallikrein inhibitors; (6) tachykinin receptor antagonists, including neurokinin₁ and neurokinin₂ receptor subtype antagonists; (7) calcitonin gene-related peptide (CGRP) receptor
20 antagonists; (8) interleukin receptor antagonists; (9) inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, including (a) phospholipase inhibitors, including PLA₂ isoform inhibitors and PLC isoform inhibitors (b) cyclooxygenase inhibitors, and (c) lipooxygenase inhibitors; (10) prostanoid receptor antagonists including eicosanoid EP-1 and EP-4 receptor subtype antagonists and
25 thromboxane receptor subtype antagonists; (11) leukotriene receptor antagonists including leukotriene B₄ receptor subtype antagonists and leukotriene D₄ receptor subtype antagonists; (12) opioid receptor agonists, including μ -opioid, δ -opioid, and κ -opioid receptor subtype agonists; (13) purinoceptor agonists and antagonists including P_{2X} receptor antagonists and P_{2Y} receptor antagonists; and (14) calcium
30 channel antagonists.

 Suitable chondroprotective agents include, for example, (1) antagonists of receptors for the interleukin1 family of proteins, including, for example, IL-1 β , IL-17 and IL-18; (2) antagonists of the tumor necrosis factor (TNF) receptor family, including, for example, TNF-R1; (3) agonists for interleukin 4, 10 and 13 receptors;
35 (4) agonists for the TGF- β receptor superfamily, including, for example, BMP-2, BMP-4 and BMP-7; (5) inhibitors of COX-2; (6) inhibitors of the MAP kinase

family, including, for example, p38 MAP kinase; (7) inhibitors of the matrix metalloproteinases (MMP) family of proteins, including, for example, MMP-3 and MMP-9; (8) inhibitors of the NF- κ B family of proteins, including, for example, the p50/p65 dimer complex with I κ B; (9) inhibitors of the nitric oxide synthase (NOS) family, including, for example, iNOS; (10) agonists and antagonists of integrin receptors, including, for example, agonists of $\alpha_v\beta_3$ integrin; (11) inhibitors of the protein kinase C (PKC) family; (12) inhibitors of the protein tyrosine kinase family, including, for example, the src subfamily; (13) modulators of protein tyrosine phosphatases; and (14) inhibitors of protein src homology 2 (SH2) domains.

Specific preferred embodiments of the solution of the present invention for use in chondroprotection and arthroscopic procedures preferably include a combination of agents that act simultaneously on distinct molecular targets to promote cartilage anabolism and inhibit unregulated or excess cartilage catabolic processes to achieve maximum inhibition of inflammatory processes and maintain cartilage homeostasis, thereby achieving a chondroprotective effect within the joint.

In each of the surgical solutions of the present invention, the agents are included in low concentrations in a liquid or fluid solution and are delivered locally in low doses relative to concentrations and doses required with conventional methods of drug administration to achieve the desired therapeutic effect. As used herein, "liquid" or "fluid" is intended to encompass pharmaceutically acceptable, biocompatible solvents, suspensions, polymerizable and non-polymerizable gels, pastes and salves. Preferably the carrier is an aqueous solution that may include physiologic electrolytes, such as normal saline or lactated Ringer's solution. It is impossible or not practical to obtain an equivalent therapeutic effect by delivering similarly dosed agents via other (i.e., intravenous, subcutaneous, intramuscular or oral) routes of drug administration since drugs given systemically are subject to first- and second-pass metabolism. The concentration of each agent is determined in part based on its receptor dissociation constant, K_d or enzyme inhibition constant, K_i . As used herein, the term dissociation constant is intended to encompass both the equilibrium dissociation constant for its respective agonist-receptor or antagonist-receptor interaction and the equilibrium inhibitory constant for its respective activator-enzyme or inhibitor-enzyme interaction. Each agent is preferably included at a low concentration of 0.1 to 10,000 times K_d or K_i , except for cyclooxygenase inhibitors, which may be required at larger concentrations depending on the particular inhibitor selected. Preferably, each agent is included at a concentration of 1.0 to 1,000 times K_d or K_i and most preferably at approximately 100 times K_d or K_i .

These concentrations are adjusted as needed to account for dilution in the absence of metabolic transformation at the local delivery site. The exact agents selected for use in the solution, and the concentration of the agents, varies in accordance with the particular application, as described below.

5 A solution in accordance with the present invention can include just a single or multiple pain and/or inflammation inhibitory agent(s), multiple chondroprotective agent(s) at least one of which is an anabolic chondroprotective agent and at least one of which is an inhibitor of cartilage catabolism, or a combination of both chondroprotective agent(s) and pain and/or inflammation inhibitory agents, at low
10 concentration. However, due to the aforementioned synergistic effect of multiple agents, and the desire to broadly block pain and inflammation, and cartilage destruction, it is preferred that multiple agents be utilized.

 The surgical solutions constitute a novel therapeutic approach by combining multiple pharmacologic agents acting at distinct receptor and/or enzyme molecular
15 targets. To date, pharmacologic strategies have focused on the development of highly specific drugs that are selective for individual receptor subtypes and enzyme isoforms that mediate responses to individual signaling neurotransmitters and hormones. Furthermore, despite inactivation of a single receptor subtype or enzyme, activation of other receptor subtypes or enzymes and the resultant signal transduction often can
20 trigger a cascade effect. This explains the significant difficulty in employing a single receptor-specific drug to block a pathophysiologic process in which multiple signaling mediators (e.g., cytokines, growth factors or eicosonoids) play a role. Therefore, targeting only a specific individual receptor subtype or isotype is likely to be ineffective.

25 In contrast to the standard approach to pharmacologic therapy, the therapeutic approach of the present surgical solutions is based on the rationale that a combination of drugs acting simultaneously on distinct molecular targets is highly effective in the inhibition of the full spectrum of events that underlie the development of a pathophysiologic state. Furthermore, instead of targeting a specific receptor subtype
30 alone, the surgical solutions are composed of drugs that target common molecular mechanisms operating in different cellular physiologic processes involved in the development of pain, inflammation, and cartilage degradation (see FIGURE 1). In this way, the cascading of additional receptors and enzymes in the nociceptive, inflammatory, and cartilage degradation pathways is minimized by the surgical
35 solutions. In these pathophysiologic pathways, the surgical solutions inhibit the cascade effect both "upstream" and "downstream".

An example of "upstream" inhibition is the cyclooxygenase antagonists in the setting of pain and inflammation. The cyclooxygenase enzymes (COX₁ and COX₂) catalyze the conversion of arachidonic acid to prostaglandin H which is an intermediate in the biosynthesis of inflammatory and nociceptive mediators including prostaglandins, leukotrienes, and thromboxanes. The cyclooxygenase inhibitors block "upstream" the formation of these inflammatory and nociceptive mediators. This strategy precludes the need to block the interactions of the seven described subtypes of prostanoid receptors with prostanoid products of the COX biochemical pathway. A similar "upstream" inhibitor included in the surgical solutions is aprotinin, a kallikrein inhibitor. The enzyme kallikrein, a serine protease, cleaves the high molecular weight kininogens in plasma to produce bradykinins, important mediators of pain and inflammation. By inhibition of kallikrein, aprotinin effectively inhibits the synthesis of bradykinins, thereby providing an effective "upstream" inhibition of these inflammatory mediators.

The surgical solutions also make use of "downstream" inhibitors to control the pathophysiologic pathways. In synoviocyte and chondrocyte preparations that have been treated with a variety of inflammatory cytokines (e.g., IL-1 β and TNF α) implicated in progressive articular cartilage degeneration, MAP kinase inhibitors produce a cartilage protective effect. The p38 MAP kinase is a point of conveyance in signaling pathways for multiple catabolic cytokines, and its inhibition prevents upregulation of multiple cellular products mediating cartilage degradation. The MAP kinase inhibitors, therefore, provide a significant advantage to the surgical solutions in the settings of joint inflammation by providing "downstream" cartilage protective effects that are independent of the physiologic combination of cytokine receptor agonists initiating the shift cartilage homeostasis.

The following is a description of suitable drugs falling in the aforementioned classes of anti-inflammation/anti-pain agents and chondroprotective agents, as well as suitable concentrations for use in solutions, of the present invention. While not wishing to be limited by theory, the justification behind the selection of the various classes of agents which is believed to render the agents operative is also set forth.

I. Inhibitors of Pain and/or Inflammation

1. Serotonin Receptor Antagonists

Serotonin (5-HT) is thought to produce pain by stimulating serotonin₂ (5-HT₂) and/or serotonin₃ (5-HT₃) receptors on nociceptive neurons in the periphery. Most researchers agree that 5-HT₃ receptors on peripheral nociceptors mediate the

immediate pain sensation produced by 5-HT (Richardson et al., 1985). In addition to inhibiting 5-HT-induced pain, 5-HT₃ receptor antagonists, by inhibiting nociceptor activation, also may inhibit neurogenic inflammation. Barnes P.J., et. al., *Modulation of Neurogenic Inflammation: Novel Approaches to Inflammatory Disease*, Trends in Pharmacological Sciences 11, pp. 185-189 (1990). A study in rat ankle joints, however, claims the 5-HT₂ receptor is responsible for nociceptor activation by 5-HT. Grubb, B.D., et. al., *A Study of 5-HT-Receptors Associated with Afferent Nerves Located in Normal and Inflamed Rat Ankle Joints*, Agents Actions 25, pp. 216-18 (1988). Therefore, activation of 5-HT₂ receptors also may play a role in peripheral pain and neurogenic inflammation.

One goal of the solution of the present invention is to block pain and a multitude of inflammatory processes. Thus, 5-HT₂ and 5-HT₃ receptor antagonists are both suitably used, either individually or together, in the solution of the present invention, as shall be described subsequently. Amitriptyline (Elavil™) is a suitable 5-HT₂ receptor antagonist for use in the present invention. Amitriptyline has been used clinically for numerous years as an anti-depressant, and is found to have beneficial effects in certain chronic pain patients. Metoclopramide (Reglan™) is used clinically as an anti-emetic drug, but displays moderate affinity for the 5-HT₃ receptor and can inhibit the actions of 5-HT at this receptor, possibly inhibiting the pain due to 5-HT release from platelets. Thus, it also is suitable for use in the present invention.

Other suitable 5-HT₂ receptor antagonists include imipramine, trazodone, desipramine and ketanserin. Ketanserin has been used clinically for its anti-hypertensive effects. Hedner, T., et. al., *Effects of a New Serotonin Antagonist, Ketanserin, in Experimental and Clinical Hypertension*, Am J of Hypertension, pp. 317s-23s (Jul. 1988). Other suitable 5-HT₃ receptor antagonists include cisapride and ondansetron. Suitable serotonin_{1B} receptor antagonists include yohimbine, N-[-methoxy-3-(4-methyl-1-piperanzinyl)phenyl]-2'-methyl-4'-(5-methyl-1, 2, 4-oxadiazol-3-yl)[1, 1-biphenyl]-4-carboxamide ("GR127935") and methiothepin. Therapeutic and preferred concentrations for use of these drugs in the solution of the present invention are set forth in Table 1.

Table 1
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Serotonin₂ Receptor Antagonists:</u>		
amitriptyline	0.1 - 1,000	50 - 500
MDL-11,939	0.1 - 1,000	50 - 500
AMI-193	0.1 - 2,000	50 - 500
desipramine	0.1 - 1,000	50 - 500
ketanserin	0.1 - 1,000	50 - 500
<u>Serotonin₃ Receptor Antagonists:</u>		
tropisetron	0.01 - 100	0.05 - 50
metoclopramide	10 - 10,000	200 - 2,000
cisapride	0.1 - 1,000	20 - 200
ondansetron	0.1 - 1,000	20 - 200
<u>Serotonin_{1B} (Human 1D_β) Antagonists:</u>		
Isamoltare	0.1 - 1,000	50 - 500
GR127935	0.1 - 1,000	10 - 500
methiothepin	0.1 - 500	1 - 100
SB216641	0.2 - 2,000	2 - 200

5 2. Serotonin Receptor Agonists

5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors are known to inhibit adenylate cyclase activity. Thus including a low dose of these serotonin_{1A}, serotonin_{1B} and serotonin_{1D} receptor agonists in the solution should inhibit neurons mediating pain and inflammation. The same action is expected from serotonin_{1E} and serotonin_{1F} receptor agonists because these receptors also inhibit adenylate cyclase.

Buspirone is a suitable 1A receptor agonist for use in the present invention. Sumatriptan is a suitable 1A, 1B, 1D and 1F receptor agonist. A suitable 1B and 1D receptor agonist is dihydroergotamine. A suitable 1E receptor agonist is ergonovine. Therapeutic and preferred concentrations for these receptor agonists are provided in

15 Table 2.

Table 2
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Serotonin_{1A} Agonists:</u>		
buspirone	1 - 1,000	10 - 200
sumatriptan	1 - 1,000	10 - 200
<u>Serotonin_{1B} Agonists:</u>		
dihydroergotamine	0.1 - 1,000	10 - 100
sumatriptan	1 - 1,000	10 - 200
<u>naratriptan</u>	1 - 1,000	10 - 200
<u>rizatriptan</u>	1 - 1,000	10 - 200
<u>zolmitriptan</u>	1 - 1,000	10 - 200
<u>L-694,247</u>	1 - 1,000	10 - 200
<u>Serotonin_{1D} Agonists:</u>		
dihydroergotamine	0.1 - 1,000	10 - 100
sumatriptan	1 - 1,000	10 - 200
<u>naratriptan</u>	1 - 1,000	10 - 200
<u>rizatriptan</u>	1 - 1,000	10 - 200
<u>zolmitriptan</u>	1 - 1,000	10 - 200
<u>L-694,247</u>	1 - 1,000	10 - 200
<u>Serotonin_{1E} Agonists:</u>		
ergonovine	10 - 2,000	100 - 1,000
<u>Serotonin_{1F} Agonists:</u>		
sumatriptan	1 - 1,000	10 - 200

3. Histamine Receptor Antagonists

- 5 Histamine receptors generally are divided into histamine₁ (H₁) and histamine₂ (H₂) subtypes. The classic inflammatory response to the peripheral administration of histamine is mediated via the H₁ receptor. Douglas, 1985. Therefore, the solution of the present invention preferably includes a histamine H₁ receptor antagonist. Promethazine (PhenerganTM) is a commonly used anti-emetic drug which potently
- 10 blocks H₁ receptors, and is suitable for use in the present invention. Interestingly, this drug also has been shown to possess local anesthetic effects but the concentrations necessary for this effect are several orders higher than that necessary to block H₁ receptors, thus, the effects are believed to occur by different mechanisms. The histamine receptor antagonist concentration in the solution is sufficient to inhibit
- 15 H₁ receptors involved in nociceptor activation, but not to achieve a "local anesthetic" effect, thereby eliminating the concern regarding systemic side effects.

Other suitable H₁ receptor antagonists include terfenadine, diphenhydramine, amitriptyline, mepyramine and tripolidine. Because amitriptyline is also effective as a serotonin₂ receptor antagonist, it has a dual function as used in the present invention. Suitable therapeutic and preferred concentrations for each of these H₁ receptor antagonists are set forth in Table 3.

Table 3

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Histamine₁ Receptor Antagonists:</u>		
promethazine	0.1 - 1,000	50 - 200
diphenhydramine	0.1 - 1,000	50 - 200
amitriptyline	0.1 - 1,000	50 - 500
terfenadine	0.1 - 1,000	50 - 500
mepyramine (pyrilamine)	0.1 - 1,000	5 - 200
tripolidine	0.01 - 100	5 - 20

4. Bradykinin Receptor Antagonists

Bradykinin receptors generally are divided into bradykinin₁ (B₁) and bradykinin₂ (B₂) subtypes. Studies have shown that acute peripheral pain and inflammation produced by bradykinin are mediated by the B₂ subtype whereas bradykinin-induced pain in the setting of chronic inflammation is mediated via the B₁ subtype. Perkins, M.N., et. al., *Antinociceptive Activity of the Bradykinin B₁ and B₂ Receptor Antagonists, des-Arg⁹, [Leu⁸]-BK and HOE 140, in Two Models of Persistent Hyperalgesia in the Rat*, Pain 53, pp. 191-97 (1993); Dray, A., et. al., *Bradykinin and Inflammatory Pain*, Trends Neurosci 16, pp. 99-104 (1993), each of which references is hereby expressly incorporated by reference.

At present, bradykinin receptor antagonists are not used clinically. Some of these drugs are peptides, and thus they cannot be taken orally, because they would be digested. Antagonists to B₂ receptors block bradykinin-induced acute pain and inflammation. Dray et. al., 1993. B₁ receptor antagonists inhibit pain in chronic inflammatory conditions. Perkins et al., 1993; Dray et. al., 1993. Therefore, depending on the application, the solution of the present invention preferably includes either or both bradykinin B₁ and B₂ receptor antagonists. For example, arthroscopy is performed for both acute and chronic conditions, and thus an irrigation solution for arthroscopy could include both B₁ and B₂ receptor antagonists.

Suitable bradykinin receptor antagonists for use in the present invention include the following bradykinin₁ receptor antagonists: the [des-Arg¹⁰] derivative of D-Arg-(Hyp³-Thi⁵-D-Tic⁷-Oic⁸)-BK ("the [des-Arg¹⁰] derivative of HOE 140", available from Hoechst Pharmaceuticals); and [Leu⁸] des-Arg⁹-BK. Suitable bradykinin₂ receptor antagonists include: [D-Phe⁷]-BK; D-Arg-(Hyp³-Thi^{5,8}-D-Phe⁷)-BK ("NPC 349"); D-Arg-(Hyp³-D-Phe⁷)-BK ("NPC 567"); and D-Arg-(Hyp³-Thi⁵-D-Tic⁷-Oic⁸)-BK ("HOE 140"). These compounds are more fully described in the previously incorporated Perkins et. al. 1993 and Dray et. al. 1993 references. Suitable therapeutic and preferred concentrations are provided in Table 4.

Table 4

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Bradykinin₁ Receptor Antagonists:</u>		
[Leu ⁸] des-Arg ⁹ -BK	1 - 1,000	50 - 500
[des-Arg ¹⁰] derivative of HOE 140	1 - 1,000	50 - 500
[Leu ⁹] [des-Arg ¹⁰] kalliden	0.1 - 500	10 - 200
<u>Bradykinin₂ Receptor Antagonists:</u>		
[D-Phe ⁷]-BK	100 - 10,000	200 - 5,000
NPC 349	1 - 1,000	50 - 500
NPC 567	1 - 1,000	50 - 500
HOE 140	1 - 1,000	50 - 500

5. Kallikrein Inhibitors

The peptide bradykinin is an important mediator of pain and inflammation, as noted previously. Bradykinin is produced as a cleavage product by the action of kallikrein on high molecular weight kininogens in plasma. Therefore kallikrein inhibitors are believed to be therapeutic in inhibiting bradykinin production and resultant pain and inflammation. A suitable kallikrein inhibitor for use in the present invention is aprotinin. Suitable concentrations for use in the solutions of the present invention are set forth below in Table 5.

Table 5
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Kallikrein Inhibitor:</u>		
Aprotinin	0.1 - 1,000	50 - 500

6. Tachykinin Receptor Antagonists

5 Tachykinins (TKs) are a family of structurally related peptides that include substance P, neurokinin A (NKA) and neurokinin B (NKB). Neurons are the major source of TKs in the periphery. An important general effect of TKs is neuronal stimulation, but other effects include endothelium-dependent vasodilation, plasma protein extravasation, mast cell recruitment and degranulation and stimulation of
 10 inflammatory cells. Maggi, C.A., *Gen. Pharmacol.*, Vol. 22, pp. 1-24 (1991). Due to the above combination of physiological actions mediated by activation of TK receptors, targeting of TK receptors is a reasonable approach for the promotion of analgesia and the treatment of neurogenic inflammation.

6a. Neurokinin₁ Receptor Subtype Antagonists

15 Substance P activates the neurokinin receptor subtype referred to as NK₁. Substance P is an undecapeptide that is present in sensory nerve terminals. Substance P is known to have multiple actions which produce inflammation and pain in the periphery after C-fiber activation, including vasodilation, plasma extravasation and degranulation of mast cells. Levine, J.D., et. al., *Peptides and the Primary*
 20 *Afferent Nociceptor*, J. Neurosci. 13, p. 2273 (1993). A suitable Substance P antagonist is ([D-Pro⁹[spiro-gamma-lactam]Leu¹⁰,Trp¹¹]physalaemin-(1-11)) ("GR 82334"). Other suitable antagonists for use in the present invention which act on the NK₁ receptor are: 1-imino-2-(2-methoxy-phenyl)-ethyl-7,7-diphenyl-4-perhydroisoindolone(3aR,7aR) ("RP 67580"); and 2S,3S-cis-3-(2-methoxybenzylamino)-2-benzhydrylquinuclidine ("CP 96,345").
 25 Suitable concentrations for these agents are set forth in Table 6.

Table 6

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Neurokinin₁ Receptor Subtype Antagonists</u>		
GR 82334	1 - 1,000	10 - 500
CP 96,345	1-10,000	100-1,000
RP 67580	0.1-1,000	100-1,000

6b. Neurokinin₂ Receptor Subtype Antagonists

- 5 Neurokinin A is a peptide which is colocalized in sensory neurons with substance P and which also promotes inflammation and pain. Neurokinin A activates the specific neurokinin receptor referred to as NK₂. Edmonds-Alt, S., et. al., *A Potent and Selective Non-Peptide Antagonist of the Neurokinin A (NK₂) Receptor*, Life Sci. 50:PL101 (1992). Examples of suitable NK₂ antagonists include: ((S)-N-methyl-N-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]-benzamide (“(±)-SR 48968”); Met-Asp-Trp-Phe-Dap-Leu (“MEN 10,627”); and cyc(Gln-Trp-Phe-Gly-Leu-Met) (“L 659,877”). Suitable concentrations of these agents are provided in Table 7.

Table 7

15 Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
<u>Neurokinin₂ Receptor Subtype Antagonists:</u>		
MEN 10,627	1-1,000	10-1,000
L 659,877	10-10,000	100-10,000
(±)-SR 48968	10-10,000	100-10,000

7. CGRP Receptor Antagonists

- 20 Calcitonin gene-related peptide (CGRP) is a peptide which is also colocalized in sensory neurons with substance P, and which acts as a vasodilator and potentiates the actions of substance P. Brain, S.D., et. al., *Inflammatory Oedema Induced by Synergism Between Calcitonin Gene-Related Peptide (CGRP) and Mediators of Increased Vascular Permeability*, Br. J. Pharmacol. 99, p. 202 (1985). An example

of a suitable CGRP receptor antagonist is I-CGRP-(8-37), a truncated version of CGRP. This polypeptide inhibits the activation of CGRP receptors. Suitable concentrations for this agent are provided in Table 8.

Table 8

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
CGRP Receptor Antagonist: I-CGRP-(8-37)	1-1,000	10-500

8. Interleukin Receptor Antagonist

Interleukins are a family of peptides, classified as cytokines, produced by leukocytes and other cells in response to inflammatory mediators. Interleukins (IL) may be potent hyperalgesic agents peripherally. Ferriera, S.H., et. al., *Interleukin-1 β as a Potent Hyperalgesic Agent Antagonized by a Tripeptide Analogue*, Nature 334, p. 698 (1988). An example of a suitable IL-1 β receptor antagonist is Lys-D-Pro-Thr, which is a truncated version of IL-1 β . This tripeptide inhibits the activation of IL-1 β receptors. Suitable concentrations for this agent are provided in Table 9.

Table 9

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Interleukin Receptor Antagonist: Lys-D-Pro-Thr	1-1,000	10-500

9. Inhibitors of Enzymes Active in the Synthetic Pathway for Arachidonic Acid
Metabolites

9a. Phospholipase Inhibitors

The production of arachidonic acid by phospholipase A₂ (PLA₂) enzymes (cPLA₂, iPLA₂, sPLA₂) and phospholipase C (PLC) results in a cascade of reactions that produces numerous mediators of inflammation, known as eicosanoids. There are a number of stages throughout this pathway that can be inhibited, thereby decreasing

the production of these inflammatory mediators. Examples of inhibition at these various stages are given below.

Inhibition of the enzyme PLA₂ isoform inhibits the release of arachidonic acid from cell membranes, and therefore inhibits the production of prostaglandins and leukotrienes resulting in decreased inflammation and pain. Glaser, K.B., *Regulation of Phospholipase A2 Enzymes: Selective Inhibitors and Their Pharmacological Potential*, Adv. Pharmacol. 32, p. 31 (1995). An example of a suitable PLA₂ isoform inhibitor is manoalide. Suitable concentrations for this agent are included in Table 10. Inhibition of the phospholipase C_γ (PLC_γ) isoform also will result in decreased production of prostanoids and leukotrienes, and, therefore, will result in decreased pain and inflammation. An example of a PLC_γ isoform inhibitor is 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

Table 10

Therapeutic and Preferred Concentrations of

15

Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Phospholipase Inhibitor:		
manoalide	100-100,000	500-10,000
aristolochic acid	40-400,000	400-40,000
ACA	10-100,000	100-10,000
HELSS	6-6,000	60-6,000

9b. Cyclooxygenase Inhibitors

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory, anti-pyretic, anti-thrombotic and analgesic agents. Lewis, R.A., *Prostaglandins and Leukotrienes*, In: Textbook of Rheumatology, 3d ed. (Kelley W.N., et. al., eds.), p. 258 (1989). The molecular targets for these drugs are type I and type II cyclooxygenases (COX-1 and COX-2). These enzymes are also known as Prostaglandin H Synthase (PGHS)-1 (constitutive) and -2 (inducible), and catalyze the conversion of arachidonic acid to Prostaglandin H which is an intermediate in the biosynthesis of prostaglandins and thromboxanes. The COX-2 enzyme has been identified in endothelial cells, macrophages, and fibroblasts. This enzyme is induced by IL-1 and TNF-α, and its expression is upregulated at sites of inflammation. Constitutive activity of COX-1 and induced activity of COX-2 both lead to synthesis of prostaglandins which contribute to pain and inflammation.

Many NSAIDs currently on the market (diclofenac, naproxen, indomethacin, ibuprofen, etc.) are generally nonselective inhibitors of both isoforms of COX, but may show greater selectivity for COX-1 over COX-2, although this ratio varies for the different compounds. Use of COX-1 and 2 inhibitors to block formation of prostaglandins represents a better therapeutic strategy than attempting to block interactions of the natural ligands with the seven described subtypes of prostanoid receptors. Reported antagonists of the eicosanoid receptors (EP-1, EP-2, EP-3) are quite rare and only specific, high affinity antagonists of the thromboxane A₂ receptor have been reported. Wallace, J. and Cirino, G. *Trends in Pharm. Sci.*, Vol. 15 pp. 405-406 (1994).

Representative therapeutic and preferred concentrations of cyclooxygenase inhibitors for use in the solution are provided in Table 11.

Table 11
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Cyclooxygenase Inhibitors:		
ketorolac	100 - 10,000	500 - 5,000
indomethacin	1,000 - 500,000	10,000 - 200,000

9c. Lipoxygenase Inhibitors

Inhibition of the enzyme lipoxygenase inhibits the production of leukotrienes, such as leukotriene B₄, which is known to be an important mediator of inflammation and pain. Lewis, R.A., *Prostaglandins and Leukotrienes*, In: Textbook of Rheumatology, 3d ed. (Kelley W.N., et. al., eds.), p. 258 (1989). An example of a 5-lipoxygenase antagonist is 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone ("AA 861"), suitable concentrations for which are listed in Table 12.

Table 12
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Lipoxygenase Inhibitor:		
AA 861	100-10,000	500-5,000

Caffeic acid	500-50,000	2,000-20,000
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10. Prostanoid Receptor Antagonists

Specific prostanoids produced as metabolites of arachidonic acid mediate their inflammatory effects through activation of prostanoid receptors. Examples of classes of specific prostanoid antagonists are the eicosanoid EP-1 and EP-4 receptor subtype antagonists and the thromboxane receptor subtype antagonists. A suitable prostaglandin E₂ receptor antagonist is 8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-acetylhydrazide ("SC 19220"). A suitable thromboxane receptor subtype antagonist is [15-[1 α , 2 β (5Z), 3 β , 4 α]-7-[3-[2-(phenylamino)-carbonyl]hydrazino] methyl]-7-oxobicyclo-[2,2,1]-hept-2-yl]-5-heptanoic acid ("SQ 29548").

Suitable concentrations for these agents are set forth in Table 13.

Table 13

Therapeutic and Preferred Concentrations of Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Eicosanoid EP-1 Antagonist: SC 19220	100-10,000	500-5,000

11. Leukotriene Receptor Antagonists

The leukotrienes (LTB₄, LTC₄, and LTD₄) are products of the 5-lipoxygenase pathway of arachidonic acid metabolism that are generated enzymatically and have important biological properties. Leukotrienes are implicated in a number of pathological conditions including inflammation. Specific antagonists are currently being sought by many pharmaceutical companies for potential therapeutic intervention in these pathologies. Halushka, P.V., et al., Annu. Rev. Pharmacol. Toxicol. 29: 213-239 (1989); Ford-Hutchinson, A. Crit. Rev. Immunol. 10: 1-12 (1990). The LTB₄ receptor is found in certain immune cells including eosinophils and neutrophils. LTB₄ binding to these receptors results in chemotaxis and lysosomal enzyme release thereby contributing to the process of inflammation.

The signal transduction process associated with activation of the LTB₄ receptor involves G-protein-mediated stimulation of phosphatidylinositol (PI) metabolism and elevation of intracellular calcium (see FIGURE 2).

An example of a suitable leukotriene B₄ receptor antagonist is SC (+)-(S)-7-(3-(2-(cyclopropylmethyl)-3-methoxy-4-[(methylamino)-

carbonyl]phenoxy(propoxy)-3,4-dihydro-8-propyl-2H-1-benzopyran-2-propanoic acid ("SC 53228"). Concentrations for this agent that are suitable for the practice of the present invention are provided in Table 14. Other suitable leukotriene B₄ receptor antagonists include [3-[-2(7-chloro-2-quinolinyl)ethenyl]phenyl] [[3-(dimethylamino-3-oxopropyl)thio] methyl]thiopropoic acid ("MK 0571") and the drugs LY 66,071 and ICI 20,3219. MK 0571 also acts as a LTD₄ receptor subtype antagonist.

Table 14

Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Leukotriene B ₄ Antagonist: SC 53228	100-10,000	500-5,000

10 12. Opioid Receptor Agonists

Activation of opioid receptors results in anti-nociceptive effects and, therefore, agonists to these receptors are desirable. Opioid receptors include the μ -, δ - and κ -opioid receptor subtypes. The μ -receptors are located on sensory neuron terminals in the periphery and activation of these receptors inhibits sensory neuron activity. Basbaum, A.I., et. al., *Opiate analgesia: How Central is a Peripheral Target?*, N. Engl. J. Med., 325:1168 (1991). δ - and κ -receptors are located on sympathetic efferent terminals and inhibit the release of prostaglandins, thereby inhibiting pain and inflammation. Taiwo, Y.O., et. al., *Kappa- and Delta-Opioids Block Sympathetically Dependent Hyperalgesia*, J. Neurosci., Vol. 11, page 928 (1991). The opioid receptor subtypes are members of the G-protein coupled receptor superfamily. Therefore, all opioid receptor agonists interact and initiate signaling through their cognate G-protein coupled receptor. Examples of suitable μ -opioid receptor agonists are fentanyl and Try-D-Ala-Gly-[N-MePhe]-NH(CH₂)-OH ("DAMGO"). An example of a suitable δ -opioid receptor agonist is [D-Pen²,D-Pen⁵]enkephalin ("DPDPE"). An example of a suitable κ -opioid receptor agonist is (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzene acetamide ("U50,488"). Suitable concentrations for each of these agents are set forth in Table 15.

Table 15
Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
μ -Opioid Agonist:		
DAMGO	0.1-100	0.5-20
sufentanyl	0.01-50	1-20
fentanyl	0.1-500	10-200
PL 017	0.05-50	0.25-10
δ -Opioid Agonist:		
DPDPE	0.1-500	1.0-100
κ -Opioid Agonist:		
U50,488	0.1-500	1.0-100

13. Purinoceptor Antagonists

5 Extracellular ATP acts as a signaling molecule through interactions with P_2 purinoceptors. One major class of purinoceptors are the P_{2X} purinoceptors which are ligand-gated ion channels possessing intrinsic ion channels permeable to Na^+ , K^+ , and Ca^{2+} . P_{2X} receptors described in sensory neurons are important for primary afferent neurotransmission and nociception. ATP is known to depolarize sensory

10 neurons and plays a role in nociceptor activation since ATP released from damaged cells stimulates P_{2X} receptors leading to depolarization of nociceptive nerve-fiber terminals. The P_{2X_3} receptor has a highly restricted distribution (Chen, C.C., et. al., Nature, Vol. 377, pp. 428-431 (1995)) since it is selectively expressed in sensory C-fiber nerves that run into the spinal cord and many of these C-fibers are known to

15 carry the receptors for painful stimuli. Thus, the highly restricted localization of expression for the P_{2X_3} receptor subunits make these subtypes excellent targets for analgesic action (see FIGURES 3 and 7).

Calcium-mobilizing purine receptors, which belong to the G-protein receptor superfamily, have been described on the surface of mammalian articular

20 chondrocytes. ATP was found to stimulate a dose-dependent, transient rise in the concentration of calcium ions in differentiated, primary chondrocytes. Heterologous desensitization experiments demonstrated that chondrocytes showed no subsequent response to UTP after initial stimulation with ATP. These results are consistent with the presence of a P_{2Y} receptors of the cell surface of chondrocytes. Purine-induced

25 calcium mobilization in passaged chondrocytes showed the same pharmacological profile with respect to agonist sensitivity. ATP and UTP did not alter cartilage

matrix synthesis as measured by rate of incorporation of [35S]sulfate into glycosaminoglycan by cartilage explants or primary chondrocytes. Matrix degradation, measured by release of glycosaminoglycan from cartilage explants, was also unaltered by either agonist. The presence of a functional P2Y purine receptor on the surface of primary articular chondrocytes enable concentrations of extracellular purines, such as ATP, to activate chondrocyte metabolism.

Other studies have defined the expression of both P1 and P2 purine receptor genes by human articular chondrocytes and profiled ligand-mediated prostaglandin E2 release. The P2Y2 receptor agonists ATP and UTP stimulated a small release of PGE2 that was synergistically enhanced after pretreatment with human IL-1 α . PGE2 release in response to coaddition of ATP and UTP after IL-1 pretreatment was mimicked by phorbol myristate acetate. The function of the P2Y2 receptor is to increase IL-1-mediated PGE2 release, thereby promoting pain and inflammation within the joint. Thus, the use of P2Y antagonists in the present invention should prevent activation of inflammatory mediator production by both synoviocytes and chondrocytes.

Suitable antagonists of P_{2X}/ATP purinoceptors for use in the present invention include, by way of example, suramin and pyridoxylphosphate-6-azophenyl-2,4-disulfonic acid ("PPADS"). Suitable concentrations for these agents are provided in Table 16.

Table 16

**Therapeutic and Preferred Concentrations of
Pain and/or inflammation Inhibitory Agents**

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
P2X and/or P2Y Antagonists:		
suramin	100-100,000	10,000-100,000
PPADS	100-100,000	10,000-100,000

14. Ca²⁺ Channel Antagonists

Calcium channel antagonists are a distinct group of drugs that interfere with the transmembrane flux of calcium ions required for activation of cellular responses mediating neuroinflammation. Calcium entry into synoviocytes and chondrocytes is a key event mediating activation of responses in these cells. Furthermore, the role of bradykinin, histamine, serotonin (SHT₂) and neurokinin receptors (NK₁ and NK₂) in

mediating the neuroinflammation signal transduction pathway includes increases in intracellular calcium, thus leading to activation of calcium channels on the plasma membrane. In many tissues, calcium channel antagonists, such as nifedipine, can reduce the release of arachidonic acid, prostaglandins, and leukotrienes that are evoked by various stimuli. Moncada, S., Flower, R. and Vane, J. in *Goodman's and Gilman's Pharmacological Basis of Therapeutics*, (7th ed.), MacMillan Publ. Inc., pp. 660-5 (1995).

- Finally, calcium channel antagonists and either tachykinin, histamine or bradykinin antagonists exhibit synergistic effects in inhibiting neuroinflammation.
- The role of neurokinin receptors in mediating neuroinflammation has been established. The neurokinin₁ (NK₁) and neurokinin₂ (NK₂) receptor (members of the G-protein coupled superfamily) signal transduction pathway includes increases in intracellular calcium, thus leading to activation of calcium channels on the plasma membrane. Similarly, activation of bradykinin₂ (BK₂) receptors is coupled to increases in intracellular calcium in synoviocytes and chondrocytes. Thus, calcium channel antagonists interfere with a common mechanism involving elevation of intracellular calcium, part of which enters through L-type channels. This is the basis for synergistic interaction between calcium channel antagonists and antagonists to neurokinin, histamine, P₂Y and bradykinin₂ receptors.
- Suitable calcium channel antagonists for the practice of the present invention include nisoldipine, nifedipine, nimodipine, lacidipine, isradipine and amlodipine. Suitable concentrations for these agents are set forth in Table 17.

Table 17

Therapeutic and Preferred Concentrations of

25

Spasm Inhibitory Agents

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Calcium Channel Antagonists:		
nisoldipine	1-10,000	100-1,000
nifedipine	1-10,000	100-5,000
nimodipine	1-10,000	100-5,000
lacidipine	1-10,000	100-5,000
isradipine	1-10,000	100-5,000
amlodipine	1-10,000	100-5,000

II. Agents For The Inhibition Of Cartilage Degradation

Recent advances in the understanding of the biochemistry and molecular biology of inflammation and cartilage destruction have implicated a role for numerous endogenous cytokines. Multiple pro-inflammatory mediators that have been implicated in producing loss of cartilage in the inflamed joint are the cytokines, TNF- α , IL-1, IL-6 and IL-8. Elevated levels of a number of these pro-inflammatory cytokines appear rapidly in the synovial fluid of acutely injured knee joints and remain elevated in patients for at least 4 weeks (Cameron, ML et al., "Synovial fluid cytokine concentrations as possible prognostic indicators in the ACL-deficient knee," *Knee Surg. Sports Traumatol. Arthroscopy* 2:38-44 (1994)). These cytokines are produced locally in the joint from several activated cell types, including synovial fibroblasts, synovial macrophages, and chondrocytes. The locally produced cytokines mediate pathophysiological events in acute and chronic inflammatory conditions and are important autocrine and paracrine mediators of cartilage catabolism. The actions of these cytokines are characterized by their ability to cause multiple effects on distinct cellular targets and by their ability to interact in either a positive or negative synergistic manner with other cytokines. IL-1 and TNF- α are particularly important since they also initiate chondrodestructive effects by disrupting the balance between the normal turnover and destruction of cartilage matrix components by modulating the activity of endogenous proteins, e.g., matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase (TIMP). Cytokine control of cartilage homeostasis represents a highly regulated balance between active mediators acting on chondrocytes which determines whether matrix degradation or repair occurs.

Injury to the joint frequently produces an inflammatory response within the joint space which involves the synovial tissue and may lead to degradation of articular cartilage. Dramatic shifts in synovial and cartilage metabolism of the human knee have been described following joint injury and arthroscopic surgery (Cameron, M.L. et al. (1994), *supra*; Cameron, M.L. et al., "The natural history of the anterior cruciate ligament-deficient knee: Changes in synovial fluid cytokine and keratan sulfate concentrations," *Am. J. Sports Med.* 25:751-754 (1997)). Specific pro-inflammatory cytokine levels increase dramatically (up to 2-4 orders of magnitude) in knee joint synovial fluids during the acute inflammatory phase seen after anterior cruciate ligament (ACL) rupture. Significant changes also occur in concentrations of cartilage matrix molecules due to overproduction of matrix metalloproteinases (MMPs), such as collagenase and stromelysin-1, which are elevated in the synovial fluid of patients after acute trauma (Lohmander, L.S. et al., "Temporal patterns of

stromelysin-1 tissue inhibitor, and proteoglycan fragments in human knee joint fluid after injury to the cruciate ligament or meniscus," *J. Orthopaedic Res.* 12:21-28 (1994)). Temporally, the changes in cytokines and cartilage matrix markers (e.g., proteoglycans) in synovial fluid, which are correlated with cartilage degeneration, are
5 maximal in the acute injury period but persist for extended periods (3 months to one year), declining slowly and remaining greater than pre-injury baseline levels.

Trauma due to arthroscopic surgery itself causes significant post-surgical inflammation which reflects additional inflammatory activation of cells in the joint, including upregulation of cyclooxygenase-2 and other pro-inflammatory cytokines. A
10 significant proportion (60-90%) of patients with rupture of the ACL show radiographic changes of the knee indicative of osteoarthritis (OA) 10-15 years after injury (Cameron, M.L. et al. (1994), *supra*). Thus, the combined effects of initial joint injury and surgical trauma may induce a sustained inflammatory state and associated changes in cartilage matrix metabolism which appear to be causative
15 factors resulting in the subsequent development of degenerative changes in articular cartilage and early development of osteoarthritis. The magnitude of this health problem is substantial since the total estimated number of arthroscopic procedures performed in the United States alone in 1996 was 1.8 million with an estimated growth rate of approximately 10% per annum. Thus, it is desirable to provide a
20 pharmaceutical method to prevent degradation of articular cartilage within the joint.

While post-surgical pain and inflammation are recognized as significant clinical problems, current pharmacological treatment regimens for arthroscopic surgery are only directed at acute postoperative analgesia. Existing surgical treatment modalities do not address the chronic inflammatory state that is induced post-
25 operatively and the need to inhibit cartilage destruction of the operatively treated joint. There is a clear need, therefore, to develop an effective, integrated drug therapy that will address both the acute and chronic aspects of pain and inflammation, as well as pathological changes in cartilage metabolism in the injured and operatively treated joint.

30 According to this aspect of the invention, a method is provided for reducing or preventing destruction of articular cartilage in a joint, by administering directly to the joint of a patient a composition which includes one or more metabolically active chondroprotective agents together with one or more agents for the inhibition of pain and/or inflammation, as previously described, or alternatively a combination of two
35 or more metabolically active chondroprotective agents, at least one of which promotes cartilage anabolic processes and at least one of which is an inhibitor of

cartilage catabolic processes, in a pharmaceutically effective carrier for intra-articular delivery. Metabolically active agents include, but are not limited to, all compounds that act directly or indirectly to modulate or alter the biological, biochemical or biophysical state of a cell, including agents that alter the electrical potential of the plasma membrane, the ligand binding or enzymatic activity of cellular receptors, intracellular or extracellularly located enzymes, protein-protein interactions, RNA-proteins interactions, or DNA-protein interactions. For example, such agents may include receptor agonists that initiate signal transduction cascades, antagonists of receptors that inhibit signalling pathways, activators and inhibitors of intracellular or extracellular enzymes and agents that modulate the binding of transcription factors to DNA.

Specifically, one aspect of the present invention provides a pharmacological method of treating the injured or operatively treated joint using a combination of cartilage protective agents delivered locally to achieve maximal therapeutic benefit. The use of a combination of chondroprotective agents overcomes the limitations of existing therapeutic approaches that rely upon on the use of a single agent to block a multifactorial cartilage destructive process in which a shift between synthesis and degradation, in favor of catabolic processes has occurred. This aspect of the invention uniquely utilizes the approach of combining of agents that act simultaneously on distinct molecular targets to promote cartilage anabolism and inhibit unregulated or excess cartilage catabolic processes to achieve maximum inhibition of inflammatory processes and maintain cartilage homeostasis, thereby achieving a chondroprotective effect within the joint. Inhibition of a single molecular target or biochemical mechanism known to induce cartilage destruction (catabolism), such as inhibiting interleukin-1(IL-1) binding to the IL-1 receptor, will likely not be optimal, since, for example, the actions of TNF- α mediated through its unique receptor shares many overlapping pro-inflammatory and cartilage catabolic functions with IL-1 and is also recognized as a major mediator of cartilage destruction in the joint. Similarly, utilizing pharmaceutical agents that only enhance cartilage anabolic processes in the absence of inhibiting catabolic processes will not optimally counteract catabolic factors present within the injured joint.

Specifically, one aspect of the present invention provides pharmaceutical compositions of metabolically active chondroprotective agents that are based upon a combination of at least two agents that act simultaneously on distinct molecular targets. In a representative embodiment, at least one agent is a cytokine or growth factor receptor agonist which directly provides anti-inflammatory activity and/or

promotes cartilage anabolic processes and at least a second agent is a receptor antagonist or enzyme inhibitor that acts to inhibit pro-inflammatory and/or cartilage catabolic processes. A representative drug combination includes at least one agent drawn from a class of anti-inflammatory/anabolic cytokines which act functionally to suppress the role of pro-inflammatory cytokines in the joint, promote cartilage matrix synthesis and inhibit matrix degradation. These receptor agonists include, but are not limited to, specific anti-inflammatory and anabolic cytokines, such as the interleukin (IL) agonists (e.g., IL-4, IL-10 and IL-13) and specific members of the transforming growth factor- β superfamily (e.g., TGF β and BMP-7), insulin-like growth factors (e.g., IGF-1) and fibroblast growth factors (e.g., bFGF). At least a second agent is drawn from a class of receptor antagonists or enzyme inhibitors that acts to inhibit and reduce the activity or the expression of a pro-inflammatory molecular target (e.g., the IL-1 receptor antagonists, TNF- α receptor antagonists, cyclooxygenase-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase (NOS) inhibitors, and nuclear factor kappaB (NF κ B) inhibitors). The metabolically active agents include both functional agonists and antagonists of receptors located on the surfaces of cells, as well as inhibitors of membrane bound or extracellularly secreted enzymes (e.g., stromelysin and collagenase). In addition, many of the agents are directed at novel targets which are the intracellularly localized enzymes and transcription factors that transduce and integrate the signals of the surface receptors, including inhibitors of the enzymes NOS, COX-2, and mitogen-activated protein kinases (MAPK) and inhibitors of protein-DNA interactions such as the transcription factor NF κ B. This method allows the integrity of cartilage to be maintained by simultaneously promoting cytokine-driven anabolic processes and inhibiting catabolic processes.

The multiple drug combination can be delivered locally by intra-articular injection or via infusion, including administration periprocedurally (ie., pre-operatively and/or intra-operatively and/or post-operatively) during surgical arthroscopic procedures, alone or coupled with post-operative sustained delivery, such as by a regulated pump delivery system or other sustained release delivery system. Sustained release delivery systems may include, but are not limited to, microparticles, microspheres or nanoparticles composed of proteins, liposomes, carbohydrates, synthetic organic compounds, or inorganic compounds. Thus, in some embodiments, the invention provides for a combination of agents to be delivered via injection or infusion, alone or together with analgesic and/or anti-inflammatory agents. The rapid onset of action achieved by direct, local delivery of the chondroprotective agents at or closely following the time of injury (e.g.,

perioperatively) has the potential to inhibit initial processes before they can trigger subsequent responses and thereby preemptively limit local tissue damage and the subsequent loss of cartilage.

Advantages of this aspect of the present invention include: 1) a combination
5 drug therapy directed to the multifactorial causes of cartilage destruction during acute and chronic conditions; 2) the combination of chondroprotective agents may be combined with anti-inflammatory and analgesic agents; 3) local delivery of the drug combination achieves an instantaneous therapeutic concentration of
10 chondroprotective agents within the joint; 4) using an irrigation solution periprocudurally provides continuous maintenance of drug levels within the joint in a therapeutically desirable range during an arthroscopic surgical procedure; 5) local delivery permits a reduction in total drug dose and dosing frequency compared to systemic delivery; 6) local site-directed delivery to the joint avoids systemic toxicity and reduces adverse effects; and 7) direct, local delivery to the joint enables use of
15 novel, pharmaceutically active peptides and proteins, including cytokines and growth factors, which may not be therapeutically useful if limited to systemic routes of administration.

1. Interleukin-1 (IL-1) Receptor Antagonists

The interleukin IL-1 exists in two forms, IL-1 α and IL-1 β , which are
20 polypeptides derived from separate gene products which share a similar spectrum of immunoregulatory and pro-inflammatory functions. IL-1 is a 17 kD polypeptide that can both act upon and be produced by a number of cell types in the joint, including synovial fibroblasts and macrophages, chondrocytes, endothelial cells and monocytes and macrophages. There is substantial evidence that IL-1 plays a pivotal role in joint
25 inflammation and in the pathophysiological loss of articular cartilage that occurs in the injured joint.

The actions of both forms of this cartilage destructive cytokine are mediated by one of two IL-1 receptors (IL-1R), type I IL-1 or type II IL-1 receptors. IL-1 receptors are structurally distinct and belong to a separate superfamily characterized
30 by the presence of immunoglobulin binding domains. These receptors bear close amino acid homology with other receptors containing immunoglobulin domains. Expression of the larger type I IL-1 receptor is present on T cells and fibroblasts while the smaller type II IL-1 receptor is present on B cells, monocytes, neutrophils, and bone marrow cells.

Type II IL-1 receptors bind IL-1 β with high affinity, but IL-1 β binding does not initiate intracellular signal transduction as it does upon binding to the type I IL-1 receptor. In contrast, the type II receptor serves as a precursor for a soluble IL-1 binding factor that has been shown to be shed from cells and this soluble receptor acts as a physiological IL-1 β antagonist. A naturally occurring IL-1 binding protein has been described which corresponds to the soluble external portion of the type II receptor.

A naturally occurring secreted, soluble ligand that binds to IL-1 receptors, alternatively referred to as the IL-1 receptor antagonist (sIL-1RA, IL-1Ra, IL-1ra), has been cloned, sequenced and found to encode a 22 kD protein. IL-1Ra competitively inhibits the binding of IL-1 α and IL-1 β to both type I and II IL-1 receptors. IL-1Ra is a pure receptor antagonist since its binding to the receptor does not activate the cellular signal transduction machinery of membrane associated IL-1 receptors. Despite high affinity binding of this protein to the IL-1Rs, a 10-100 fold molar excess is required to inhibit IL-1 biological responses of cells that express the type I IL-1R. Cells known to produce IL-1Ra include monocytes, neutrophils, macrophages, synoviocytes and chondrocytes. IL-1Ra has been shown to inhibit PGE₂ synthesis, induction of pro-inflammatory cytokines and MMPs, and nitric oxide production. Secreted IL-1Ra is released *in vivo* during experimentally induced inflammation. Importantly, IL-1Ra is expressed in synovial tissue and is present in normal human synovial fluid. In patients with knee injuries, levels of IL-1Ra in the synovial fluid dramatically increase in the acute phase after injury, and subsequently decrease to below normal levels in sub-acute and chronic states. Thus, the IL-1Ra has been shown to play a physiological role in responses of the joint to injury.

IL-1 is considered the dominant cartilage destructive cytokine that plays a pivotal role in joint destruction due to its ability to stimulate the production of degradative enzymes and pro-inflammatory cytokines by both chondrocytes and synoviocytes. Moreover, IL-1 β is a potent inhibitor of proteoglycan and collagen synthesis by chondrocytes. At the cellular level, IL-1 β -induced responses of synovial fibroblasts include increased production of PGE₂, collagenase and other neutral proteases and the upregulation of pro-inflammatory cytokines, IL-6 and IL-8.

IL-1, which is present in the joint fluid of patients with arthritic diseases, stimulates chondrocytes to: 1) synthesize elevated amounts of enzymes such as stromelysin, fibroblast and neutrophil collagenase and plasminogen activator, and 2) inhibit synthesis of plasminogen activator inhibitor-1 and TIMP. In addition, IL-1 β is a potent inhibitor of the synthesis of matrix constituents such as type II collagen,

the predominant form of collagen in articular cartilage, and proteoglycans. The imbalance between the levels of inhibitors and proteases leads to an increase in the amount of active proteases. This increase, combined with a suppression of matrix biosynthesis, results in degradation of cartilage. In animal studies, injection of IL-1 into rabbit knee joints causes depletion of proteoglycan from the articular cartilage.

Since IL-1 is one of the key cytokines involved in the pathogenesis of chronic synovitis and cartilage degradation, reducing its production or blocking its action represents an appropriate strategy for new treatments to reduce synovial inflammation and to provide a chondroprotective effect. A variety of therapeutic approaches for antagonizing the interaction of the agonist, IL-1, with its natural membrane bound receptor can be utilized which include: 1) naturally occurring specific inhibitors of IL-1 activity that have been characterized to date, including IL-1Ra and soluble IL-1 receptors; 2) anti-IL-1 Abs; and 3) small molecule antagonists which may be either peptidic or nonpeptidic.

The ability to block the actions of this key cytokine will have effects on many cell types in the joint (e.g., synovial fibroblasts and chondrocytes), thus inhibiting subsequent pathological effects such as infiltration of inflammatory cells into the joint, synovial hyperplasia, synovial cell activation, as well as cartilage breakdown and inhibition of cartilage matrix synthesis. An IL-1 receptor antagonist should block the propagation of the inflammatory response by IL-1 and thereby interrupt the disease process. The therapeutic potential of a number of IL-1 receptor antagonists have been established in animal models of inflammation and arthritis (RA and OA). Patients suffering from RA have improved clinically following a subcutaneous injection of IL-1Ra or an intra-articular injection of soluble Type I IL-1R.

The effects of IL-1 β and IL-1Ra depend on their respective local concentrations. In the supernatants of RA synovium pieces, IL-1 β levels were threefold higher than those of IL-1Ra. Thus, the spontaneous local production of IL-1Ra is not sufficient to inhibit IL-1 β effects because a larger (10 to 100-fold) molar excess of IL-1Ra is required to inhibit IL-1-induced biological responses in cells that express type I IL-1R. Thus, high doses of IL-1Ra have been used *in vivo* to block IL-1 in human volunteers in patients with RA. IL-1Ra present locally in the synovium provides a negative signal, down-regulating at least part of the IL-1-mediated processes in synovitis, such as leukocyte accumulation in the inflamed tissue, PGE₂ production and collagenase production by synovial cells. A chondroprotective effect of IL-1Ra has been demonstrated using direct injection of IL-1Ra into the joint in a

canine ACL model and by employing a gene therapy approach based upon transfection of the IL-1Ra gene into human synovial fibroblasts.

The present invention discloses the local delivery of an IL-1 soluble receptor protein, which is comprised of an extracellular domain of a IL-1R, and which is capable of binding an IL-1 cytokine molecule in solution. In particular, and by way of example, a soluble human IL-1 receptor (shuIL-1R) polypeptide comprising essentially the amino acid sequence 1-312, as disclosed within US Patent No. 5,319,071 and US Patent No. 5,726,148, is disclosed herein for use in combination with one or more drugs chosen from either an anti-inflammatory class, anti-pain class, or chondroprotective class. Alternatively, the local delivery of a fusion protein consisting of the sIL-1R binding domain polypeptide is proposed for use to promote chondroprotection, as disclosed in US Patent 5,319,071. In addition, the local delivery of an IL-1 receptor antagonist as disclosed within US Patent 5,817,306 is disclosed for use in the present invention. The shuIL-1R soluble receptor has been shown to bind IL-1 with nanomolar affinity. Local delivery of a therapeutically effective concentration of an IL-1R soluble receptor, such as shuIL-1R, may occur by direct injection of the joint or in an irrigation solution (e.g., during an arthroscopic surgical procedure) in combination with one or more chondroprotective drugs, anti-inflammatory drugs, or anti-pain drugs and is disclosed herein as a cartilage protective agent when applied locally to tissues of the joint in a variety of inflammatory or pathophysiological conditions. Such treatment will preemptively inhibit IL-1 stimulation of production of collagenase-1 and stromelysin-1. Employing a wholly different method based on gene delivery for local production of type 1 soluble receptors for IL-1 and/or TNF- α , it has been found that the presence of soluble receptors for these cytokines are able to confer protection to the rabbit knee joint during the acute inflammatory phase of antigen induced-arthritis.

IL-1 receptor antagonist peptides (11-15 amino acids) that bind specifically with high affinity to the human type I IL-1 receptor are suitable for use in the present invention as chondroprotective agents. These small peptides provide a number of advantages over larger recombinant IL-1 soluble receptors or recombinant IL-1ra, including ease and cost of synthesis and the ability to penetrate biological barriers. Two of the most potent peptides, based on *in vitro* efficacy are: Ac-FEWTPGWYQJYALPL-NH₂ (AF12198, IC₅₀=0.5-2nM) and Ac-FEWTPGWYQJY-NH₂ (AF11567). AF11567 is a truncated version of AF12198, lacking the 4 C-terminal residues and exhibiting slightly lower affinity for the type I IL-1 receptor but possessing a similar plasma half-life of 2.3-2.6 hrs. Poor solubility and rapid

metabolism appeared to limit the *in vivo* efficacy of AF12198 when administered systemically via intravenous infusion. These limitations are in part overcome through direct, local delivery methods such as injection into the intra-articular joint space or by inclusion in the surgical irrigation fluid or other infusion, as described within this invention. Examples of IL-1 receptor antagonist agents suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 18
Therapeutic and Preferred Concentrations of
Interleukin-1 Receptor Antagonists

<u>Compound</u>	<u>Therapeutic Concentrations (nM)</u>	<u>Most Preferred Concentrations (nM)</u>
rshuIL-1R	0.2-2000	200
rhIL-1ra	0.2-2000	200
anti-IL1-antibody	0.2-2000	200
AF12198	0.2-2000	200
AF11567	0.2-2000	200

15 2. Tumor Necrosis Factor (TNF) Receptor Antagonists

TNF- α , a cytokine mainly produced by activated macrophages, has many biological actions including transcriptional regulation of several genes that are mediated by specific TNF receptors, as well as immunoregulatory activities. Originally, two different receptors termed TNF-R1 and TNF-R2 were cloned and characterized and also found to be produced as soluble receptors.

Receptors in this family are single transmembrane proteins with considerable homology in their extracellular domains whereas their relatively short intracellular domains bear very little sequence homology. The actions of TNF are the result of the factor binding to cell surface receptors that are present on virtually all cell types that have been studied. Two receptors have been identified and cloned. One receptor type, termed TNFR-II (or Type A or 75kDa) encodes a transmembrane protein of 439

amino acids and has an apparent molecular weight of 75kDa. The second receptor type, termed TNFR-I (or Type B or 55 kDa) shows an apparent molecular weight of 55 kDa and encodes a transmembrane protein of 426 amino acids. TNFR1 contains an intracellular domain which can initiate signalling through the NF- κ B pathway.

5 Both of the TNF receptors exhibit high affinity for binding TNF α . Soluble TNF receptors (sTNFR) have been isolated and proved to arise as a result of shedding of the extracellular domains of the membrane bound receptors. Two types of sTNFR have been identified and designated as sTNFR1 (TNF BPI) and sTNFRII (TNF BPPII). Both of these soluble receptor forms have been shown to represent the truncated
10 forms of the two types of TNFR described above.

TNF- α plays a central role in the sequence of cellular and molecular events underlying the inflammatory response and cartilage destruction. Many of the effects of TNF- α overlap with the pro-inflammatory effects of IL-1. Among the pro-inflammatory actions of TNF- α is its stimulation of the release of other pro-inflammatory cytokines including IL-1, IL-6 and IL-8. TNF- α also induces the
15 release of matrix metalloproteinases from neutrophils, fibroblasts and chondrocytes that degrade cartilage, in part through the stimulation of collagenase. Furthermore, TNF- α upregulates COX-2 in normal human articular chondrocytes and synovial fibroblasts, resulting in increased PGE2 production.

20 This cytokine, along with IL-1, is considered to initiate and produce pathological effects on cartilage in the joint, including leukocyte infiltration, synovial hyperplasia, synovial cell activation, cartilage breakdown and inhibition of cartilage matrix synthesis. In particular, during synovial inflammation, increased levels of TNF- α are found in synovial fluid of joints and increased production of TNF- α by
25 synovial cells occurs. Thus, local delivery of a soluble TNF- α receptor in an irrigation solution, infusion, or injection will bind free TNF- α and function as an antagonist of TNF receptors in the surrounding tissue, thus providing a cartilage protective effect.

The present invention describes the use of functional antagonists of TNF- α
30 that act extracellularly to block interaction of the ligand with their cognate membrane receptors either by scavaging of available free ligand or by direct competitive interaction with the receptor itself, alone or in combination with other agents to provide a chondroprotective effect. A variety of therapeutic approaches for antagonizing the interaction of the agonist, TNF- α , with its natural membrane bound
35 receptor can be utilized which include: 1) the use of naturally occurring specific inhibitors of TNF- α activity that have been characterized to date, including soluble

TNF- α receptors; 2) the use of anti- TNF- α antibodies and 3) the use of small molecule antagonists which may be either peptidic or nonpeptidic.

The present invention discloses the use of a chimeric soluble receptor (CSR) protein, in which the extracellular domain of a TNF receptor, which possesses
5 binding activity for a TNF molecule, is covalently linked to a domain of an IgG molecule. In particular, and by way of first example, a chimeric polypeptide (recombinant chimera) comprising the extracellular domain of the TNF receptor extracellular polypeptide coupled to the CH2 and CH3 regions of a mouse IgG1 heavy chain polypeptide could be used, as disclosed in US Patent No. 5,447,851. The
10 chimeric TNF soluble receptor (also termed the "chimeric TNF inhibitor" in US Patent No. 5,447,851) has been shown to bind TNF- α with high affinity and has been demonstrated to be highly active as an inhibitor of TNF- α biological activity. In addition, a second example is a chimeric fusion construct comprised of the ligand binding domain of the TNF receptor with portions of the Fc antibody (termed Fc
15 fusion soluble receptors) that have been created for TNF- α receptors. The present invention also discloses the use of a soluble TNF receptor: Fc fusion protein, or any modified forms, as disclosed in US Patent No. 5,605,690. The molecular form of the active soluble receptor fusion protein can be either monomeric or dimeric. Existing studies establish that such a soluble TNF receptor:Fc fusion protein retains high
20 binding affinity for TNF- α .

Within the context of defining soluble receptors as pharmacological antagonists, the term soluble receptors includes, but is not limited to: (1) soluble receptors which correspond to naturally (endogenous) produced amino acid sequences or soluble fragments thereof consisting of an extracellular domain of full-
25 length membrane receptor, (2) recombinant soluble receptors which are truncated or partial sequences of the full length, naturally occurring receptor amino acid sequences which retain the ability to bind cognate ligand and retain biological activity and analogs thereof, and (3) chimeric soluble receptors which are recombinant soluble receptors comprised of truncated or partial sequences corresponding to a portion of
30 the extracellular binding domain of the full length receptor amino acid sequences attached through oligomers (e.g., amino acids) to a sequence corresponding to a portion of an IgG polypeptide (e.g., IgG hinge and Fc domain) which retain biological activity and the ability to bind cognate ligand.

Soluble, extracellular ligand-binding domains of cytokine receptors occur
35 naturally in body fluids and are thought to be involved in the regulation of the biologic activities of cytokines. The naturally occurring existence of soluble,

truncated forms of a number of hematopoietic cytokine receptors has been reported (IL-1R, IL-4R, IL-6R, TNFR). For example, soluble TNFR is found at concentrations of about 1-2 ng/ml in the serum and urine of healthy subjects. Lacking signal transduction functions, these cytokine binding proteins arise as a result of alternative splicing of the mRNA for the complete receptor sequence (membrane-bound form) or as a result of proteolytic cleavage and release of the membrane-bound form of the receptor. Although the *in vivo* functions of these soluble truncated receptors are not fully established, they appear to act as physiological antagonists of their complementary endogenous cytokines. This antagonism occurs because (1) scavenging of the free ligand through binding to its cognate soluble receptor reduces the effective free concentration available to the membrane-bound receptors and (2) actions of the cytokines are only produced subsequent to binding to cell surface receptors.

The TNF- α soluble receptor will function as a natural antagonist of the TNF-R1 and TNF-R2 by competing with these cell surface receptors for common pool of free ligand. Pharmacologically, the TNF soluble receptor will function as an antagonist through its ability to decrease free ligand bioavailability rather than by a mechanism of competitive inhibition (i.e., competing with an endogenous ligand for a common binding site on a membrane receptor). Addition of a therapeutically effective amount of the TNF soluble receptor to the joint should effectively neutralizing the biological activity of the ligand. Experiments in which recombinant soluble receptors have been administered *in vivo* have demonstrated the capacity to inhibit inflammatory responses and act as antagonists.

In this invention, agents suitable as chondroprotective agents for use in combination with other chondroprotective, anti-pain and/or anti-inflammatory agents to inhibit cartilage destruction include soluble TNFR, the human chimeric polypeptide (recombinant chimera) comprising the extracellular domain of the TNF- α receptor (p80) linked to the Fc portion of human IgG1, and the anti-TNF- α antibody. For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 19
Therapeutic and Preferred Concentrations TNF-Receptor Antagonists

<u>Compound</u>	Therapeutic Concentrations (nM)	Most Preferred Concentrations (nM)
sTNFR	0.1-2000	200
chimeric rhTNFR:Fc	0.1-2000	200
anti-TNF- α antibody	0.2-2000	200

3. Interleukin Receptor Agonists

5 Some cytokines are signaling glycoproteins that are important mediators of synovial inflammation and cartilage destruction. Recent analysis of the mechanism of cartilage destruction suggests that not only is the absolute level of pro-inflammatory master cytokine, IL-1, important in determining loss of cartilage, but that cytokine control of cartilage homeostasis is governed by the balance of catabolic and anabolic regulatory cytokines, and anabolic growth factors. If the balance between IL-1 β and IL-1Ra production is altered in the inflammatory state in favor of IL-1 β , then it will contribute to the pathogenesis of chronic inflammatory conditions and cartilage destruction, such as is known to occur after knee joint surgery. Potential therapeutic agents that would inhibit production of the pro-inflammatory cytokines at the sites of inflammation within the joint include the anti-inflammatory cytokines, IL-4, IL-10, and IL-13. These cytokines have been observed to greatly reduce articular cartilage destruction *in vitro* and *in vivo* via their effect on a range of pathways that reduce the impact of IL-1. Thus, anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, may be useful in reducing inflammation by: 1) reducing the production of pro-inflammatory cytokines, and 2) inducing the production of natural anti-inflammatory cytokines such as IL-1Ra, as recently demonstrated *in vivo* for IL-4.

IL-4 appears to attenuate the inflammatory process in the synovium of rheumatoid arthritis (RA) patients. In rheumatoid synovium, IL-4 has been shown to inhibit the production of pro-inflammatory cytokines by pieces of synovium, to inhibit proliferation of synoviocytes and decrease bone resorption. IL-4 may promote a direct chondroprotective effect through suppression of matrix metalloproteinase-3 (MMP-3) synthesis in human articular chondrocytes. A cell culture system employing human articular chondrocytes was used to evaluate the effect of IL-4 on

IL-1-induced production of MMP-3 and tissue inhibitor of metalloproteinase-1 (TIMP-1). It was found that IL-4 suppressed IL-1-stimulated MMP-3 protein and enzyme activity. In addition, IL-4 suppressed IL-1-induced MMP-3 mRNA. Induction of iNOS can be inhibited by IL-4, IL-10 and IL-13. Thus, IL-4 may be characterized as a protective mediator of joint destruction seen in inflammatory joint diseases.

Furthermore, the effects of IL-4 on the balance of IL-1 regulatory cytokine levels have also been found to support a cartilage protective role. IL-4 and IL-10 were found to suppress the production of inflammatory cytokines by freshly prepared rheumatoid synovial cells. While each interleukin was effective alone, the combination of IL-4 and IL-10 synergistically inhibited the IL-1 and TNF- α stimulated production of IL-6 and IL-8, without effects on cell viability. The addition of IL-4 to RA synovium cultures increased the production of IL-1Ra and decreased that of IL-1 β . *In vivo* treatment with IL-4 has recently been reported to promote a reduction in rat experimental arthritis by acting differentially on the IL-1 β /IL-1Ra balance. IL-13, another cytokine that shares many properties with IL-4, also induced IL-1Ra in RA synovium. Therefore, the local delivery of an IL-4 and IL-13 combination may provide a synergistic therapeutic value.

IL-10 has a number of properties that indicate that it is a good candidate to inhibit cartilage destruction. It inhibits both IL-1 and TNF- α release and stimulates TIMP-1 production while inhibiting MMP-2. The production of IL-10 inside the RA synovium has recently been reported and anti-inflammatory effects of IL-10 have been characterized. IL-10 suppressed IL-1 β production in an *ex vivo* RA model using pieces of synovium, but to a lesser extent than IL-4.

A protective effect of IL-4 and IL-10 treatment on cartilage destruction has been found in animal models of arthritis employing non-local methods of delivery for the cytokines. In a murine collagen-induced arthritis model, combination treatment of IL-4 and IL-10 produced substantial improvement. In addition to suppression of macroscopic signs of inflammation, combined treatment with IL-4 and IL-10 also reduced cellular infiltrates in the synovial tissue and caused pronounced protection against cartilage destruction. Moreover, levels of mRNA for TNF- α and IL-1 were highly suppressed both in the synovial tissue and in the articular cartilage. In contrast, levels of IL-1 receptor antagonist (IL-1Ra) mRNA remained elevated, which suggests that the mechanism of protection may be related to suppressed production of TNF- α and IL-1, with concomitant up-regulation of the IL-1Ra/IL-1 balance. These data are consistent with a dominant role of IL-10 in the endogenous suppression of

the inflammatory response and destruction of articular cartilage, and a combined treatment with IL-4 and IL-10 appears of potential therapeutic value.

The role of endogenous IL-4 and IL-10 and the therapeutic effect of addition of these cytokines on joint inflammation and cartilage destruction in the early stages of the macrophage dependent murine streptococcal cell wall (SCW) arthritis model have also been investigated. It was demonstrated that endogenous IL-10 plays a role in the regulation of SCW arthritis. Addition of exogenous IL-10 further enlarged the suppressive effect of endogenous IL-10. An even more pronounced effect was found with the combination of IL-4 and IL-10. The combination resulted in a reduced swelling and an increase in chondrocyte proteoglycan synthesis. Treatment with the combination of IL-4 and IL-10 substantially diminished levels of TNF- α , as occurs for IL-10 treatment alone, but also resulted in strongly reduced IL-1 β levels in the synovium, an added effect of potential clinical benefit. Overall, the data is consistent with a role for both IL-4 and IL-10 as chondroprotective agents delivered locally to joints to prevent cartilage destruction, and indicates a combination containing IL-4 and IL-10 may provide a greater potential therapeutic value than either agent alone.

Severe combined immunodeficient (SCID) mice were used as a model to assess the effect of IL-4 or IL-10 injection on cartilage degradation and mononuclear cell (MNC) recruitment to human rheumatoid synovium *in vivo*. Human rheumatoid synovium and cartilage from five rheumatoid arthritis patients were injected with recombinant human IL-4 (rhIL-4, 100 ng; rhIL-10, 100 ng), a combination of IL-4 and IL-10, or TNF-alpha (1000 U), or phosphate-buffered saline twice a week for 4 weeks. It was found that a combination of human IL-4 and IL-10 inhibited cartilage degradation and invasion by human synovial tissue, establishing the chondroprotective properties of these interleukin agonists.

Human IL-13 has been cloned and sequenced and has been found to share many of the properties of IL-4. IL-13 is about 25% homologous to IL-4. Like IL-4, IL-13 decreases the production of pro-inflammatory cytokines, including IL-1 and TNF- α , by synovial fluid mononuclear cells. IL-13 exhibits anti-inflammatory effects *in vivo* and thus has therapeutic potential in the treatment of cartilage destruction in the joint.

Compounds useful as IL-4, IL-10 and IL-13 agonists include naturally occurring human IL-4, IL-10 and IL-13, human recombinant IL-4 (rhIL-4), rhIL-10, and rhIL-13 as well as partial sequences thereof, or peptide sequences which have been constructed using recombinant DNA techniques to recognize the IL-4, IL-10 and IL-13 receptors and are capable of activating these receptors on a cell surface.

This specifically includes multispecific molecules comprised of an anti-Fc receptor portion and an anti-IL-4, anti-IL-10, and anti-IL-13 receptor portion, wherein at least one portion is constructed using recombinant DNA techniques. Within the context of defining interleukin agonists as pharmacological agonists, the term interleukin agonist includes, but is not limited to: (1) peptide sequences which correspond to naturally (endogenous) produced amino acid sequences or fragments thereof, (2) recombinant interleukins which are truncated or partial sequences of the full length naturally occurring interleukin amino acid sequences which retain the ability to bind cognate receptor and retain biological activity and analogs thereof, and (3) chimeric interleukins which are recombinant polypeptides comprised of truncated or partial sequences corresponding to a portion of the of the full length amino acid sequences attached through oligomers (e.g., amino acids) to a sequence corresponding to a portion of an IgG polypeptide (e.g., IgG hinge and Fc domain) which retain the ability to bind the cognate receptor and retain biological activity.

Examples of interleukin agonists suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 20

Therapeutic and Preferred Concentrations

<u>Compounds</u>	<u>Interleukin Agonists</u>	
	<u>Therapeutic Concentrations (nanomolar)</u>	<u>Preferred Concentrations (nanomolar)</u>
rhIL-4	0.5-5,000	5-500
rhIL-10	0.5-5,000	5-500
rhIL-13	0.5-5,000	5-500

4. Transforming Growth Factor- β Superfamily Agonists

Transforming growth factor- β (TGF- β) subfamily members are 25 kD pleiotropic, multifunctional proteins capable of influencing a variety of cellular functions and are known to be involved in tissue repair and remodeling. In many cases, it enhances the cell interaction with the extracellular matrix (ECM) and increases accumulation of ECM by stimulating production and secretion of ECM

proteins and protease inhibitors. TGF- β also has been shown to have synergistic interactions with other cytokines, generally showing anti-inflammatory activities. Multiple isoforms of TGF- β have been identified which share close amino acid sequence homologies. TGF- β 1, TGF- β 2, and TGF- β 3 have been found in human
5 tissue and are active in mammalian cells, although differing in binding affinity.

Members of the TGF- β subfamily are potent modulators of chondrocyte proliferation, differentiation and extracellular matrix accumulation. In cartilage organ cultures, TGF- β 1 regulates metabolism of proteoglycans and stimulates collagen and glycosaminoglycan synthesis by rabbit articular chondrocytes. In
10 addition, TGF- β 1 increases TIMP expression in human articular chondrocytes and down-regulates expression of IL-1 receptors in articular cartilage.

Bone morphogenetic proteins (BMPs) are multifunctional regulators of cell growth, differentiation and apoptosis that belong to the transforming growth factor (TGF)- β superfamily. More than a dozen members of the BMP protein family have
15 been identified in mammals, which can be subclassified into several groups depending on their structures. BMP-2 and BMP-4 are highly similar to each other. BMP-5, BMP-6, osteogenic protein (OP)-1 (also called BMP-7), and OP-2/BMP-8 are structurally similar to each other. Growth-differentiation factor (GDF)-5 (also
20 termed cartilage-derived morphogenetic protein-1), GDF-6 (also cartilage-derived morphogenetic protein-2), and GDF-7 form another related group. In contrast to BMP-2, BMP-4, BMP-6, and OP-1/BMP-7, which induce bone and cartilage formation *in vivo*, GDF-5, GDF-6, and GDF-7 more efficiently induce cartilage and tendon-like structures *in vivo* (Wolfman et al., 1997).

Members of the TGF- β superfamily exert their effects via binding to two
25 types of serine/threonine kinase receptors, both of which are essential for signal transduction (Massague, 1998). The type II receptors are constitutively active kinases, which transphosphorylate type I receptors upon ligand binding. The type I receptors activate intracellular substrates such as Smad proteins and it is through this mechanism that specificity of intracellular signal transduction occurs. Seven
30 different type I receptors have been isolated in mammals, which were originally termed activin receptor-like kinase (ALK)-1-ALK7. BMP type IA receptor (BMPRI-IA or ALK-3) and BMP type IB receptor (BMPRI-IB or ALK-6) are structurally similar to each other and specifically bind BMPs together with type II receptors. ALK-2 has been shown to bind activin, but recent data revealed that it is a type I
35 receptor for certain BMPs, e.g., OP-1/BMP-7 (Macias-Silva et al., 1998). ALK-1 is structurally highly similar to ALK-2, but its physiological ligand is still unknown.

ALK-5 and ALK-4 are type I receptors for TGF- β (T β R-I) and activin (ActR-IB), respectively. ALK-7 is structurally similar to ALK-4 and ALK-5, but its ligand has not been determined yet.

5 Naturally occurring TGF- β and BMP agonists as well as synthetic or human recombinant (rh) agonists suitable for use in the cartilage-protective solution of the present invention may interact with any of the BMP receptors described above. As used herein, the term "TGF- β and BMP agonists" includes fragments, deletions, additions, amino acid substitutions, mutations, and modifications thereof which retain the biological characteristics of the naturally occurring human TGF- β and BMP
10 agonist ligands. The TGF- β or BMP agonists may be used alone or in synergistic combination with other members of the TGF- β superfamily as anabolic cartilage agents (chondrogenic or promoting cartilage matrix repair) or in combination with inhibitory agents that block cartilage catabolism.

Type I receptors function as downstream components of type II receptors.
15 The specificity of the intracellular signals by type I receptors is determined by a specific region in the serine/threonine kinase domain, termed the L45 loop. Thus, the structures of the L45 loop of BMPR-IA/ALK-3 and BMPR-IB/ALK-6 (BMPR-I group) are identical to each other, and they may transduce similar signals in cells. Similarly, the L45 loops of T β R-I/ALK-5, ActR-IB/ALK-4, and ALK-7 (T β R-I
20 groups) are identical to each other, and they activate similar substrates (Chen et al., 1998). The L45 loops of ALK-1 and ALK-2 (ALK-1 group) are most divergent from the other type I receptors, but they activate substrates similar to that of the type I receptors of the BMPR-I group (Armes et al., 1999).

Various proteins may transduce signals from the TGF- β and BMP
25 serine/threonine kinase receptors. Among them, the best-studied molecules are proteins of the Smad family. Eight different Smad proteins have been identified in mammals, and these proteins are classified into three subgroups, i.e., receptor-regulated Smads (R-Smads), common partner Smads (Co-Smads), and inhibitory Smads. R-Smads are directly activated by type I receptors, from complexes with Co-
30 Smads, and translocate into the nucleus. The Smad heteromers bind to DNA directly and indirectly via other DNA-binding proteins and thus regulate the transcription of target genes. Smad1, Smad5, and Smad8 are activated by BMPs, whereas Smad2 and Smad3 are activated by TGF- β . For example, Smad2, in combination with Smad4, that functions as a Co-Smad, is translocated to the nucleus where it activates the
35 transcription of genes that mediate the biological effects of TGF. Smad6 and Smad7 are structurally distantly related to the other Smads and act as inhibitory Smads. It

has been shown that BMPs induce new cartilage and bone formation *in vitro* and *in vivo* and regulate chondrocyte growth and differentiation. Furthermore, these proteins are also implicated in the cartilage repair process. Various studies have shown that BMPs also promote and maintain the chondrogenic phenotype, which is indicated by their ability to stimulate proteoglycan synthesis in chick limb bud cells culture and fetal rat chondroblasts, as well as in rabbit and bovine articular chondrocytes. The importance of BMPs for cartilage and bone formation has been proven by transgenic approach in which specific BMP gene knockouts were studied.

One member of the BMP family, osteogenic protein (OP-1 or BMP-7), appears particularly important for cartilage homeostasis under normal and pathological conditions, such as during repair of cartilage. OP-1 appears to be the only member of the BMP family, along with cartilage-derived morphogenetic proteins, which is expressed by adult articular chondrocytes (Chubinskaya, S., *J. Histochemistry and Cytochemistry* 48: 239-50 (2000)). OP-1 was originally purified from bone matrix and shown to induce cartilage and bone formation. The human OP-1 gene has been cloned and biologically active recombinant OP-1 homodimers have been produced. Human recombinant OP-1 can stimulate synthesis of aggrecan and collagen Type II by human articular chondrocytes *in vitro*. It can also counteract the deleterious effects of IL-1 on the metabolism of these chondrocytes and block bovine cartilage damage mediated by fibronectin fragments. This effect was demonstrated by studying the effects of recombinant human OP-1 on the production of proteoglycan, prostaglandin E2, and IL-1 receptor antagonist by human articular chondrocytes cultured in the presence of interleukin-1beta. Treatment of human articular chondrocytes with OP-1 was effective in overcoming the down-regulation of proteoglycan synthesis induced by low doses of IL-1 β . Furthermore, a study found that OP-1 stimulates the synthesis of hyaluronan and CD44, other molecules required for matrix assembly by human chondrocytes. These studies of the expression and regulation to OP-1 in human adult cartilage suggest a role for OP-1 in cartilage protection and repair and indicate that OP-1 can be used as a therapeutic agent that promotes cartilage anabolism and repair of human articular cartilage.

OP-1 (BMP-7) induces cartilage and bone formation when implanted at intra- and extraskeletal sites *in vivo*. The influence of OP-1 on healing of full-thickness articular cartilage defects was investigated by drilling two adjacent holes through articular cartilage of rabbit knee joint. OP-1 induced articular cartilage healing and regeneration of the joint surface that contained cells resembling mature joint chondrocytes.

These data suggest that one preferred embodiment of the solution useful for the practice of the present invention for the prevention of cartilage degradation and maintaining biological homeostasis of articular cartilage in humans after surgical trauma could include local application of a member of the TGF- β superfamily, preferably either TGF β 2, BMP-7 (OP-1) or BMP-2, or an equivalent agonist which acts through the same receptors employed by these ligands. The local delivery may occur in combination with a drug or drugs that are inhibitors of cartilage catabolic processes (eg. such as MAP kinase inhibitors, MMP inhibitors or nitric oxide synthase inhibitors) and/or other agents for the inhibition of pain and inflammation.

Within the context of defining TGF- β and BMP agonists as pharmacological agonists, the term TGF- β and BMP agonist includes, but is not limited to: (1) peptide sequences which correspond to naturally (endogenous) produced amino acid sequences or fragments thereof, (2) recombinant TGF- β s and BMPs which are truncated or partial sequences of the full length naturally occurring TGF- β and BMP amino acid sequences which retain the ability to bind cognate their respective receptor and retain biological activity and analogs thereof, and (3) chimeric TGF- β s and BMPs which are recombinant polypeptides comprised of truncated or partial sequences corresponding to a portion of the of the full length amino acid sequences attached through oligomers (e.g., amino acids) to a sequence corresponding to a portion of an IgG polypeptide (e.g., IgG hinge and Fc domain) which retain the ability to bind the cognate receptor and retain biological activity.

Examples of TGF- β and BMP agonists suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

A range of therapeutic concentrations for delivery in the surgical solution to the joint may be estimated from values of the dissociation constants (K_d) of each ligand for its cognate receptor. While these values will vary for particular cell types and tissues, the following example is given for BMP-4. Binding experiments with 125 I-BMP-4, revealed the presence of specific, high-affinity binding sites with an apparent dissociation constant of 110 pM and about 6000 receptors per cell. Therefore, at 11 nM BMP-4, binding of the ligand will be maximal and the available receptors will be fully occupied (saturated). The presence of functional receptors for BMP-4 on primary articular chondrocytes has been demonstrated.

Table 21
Therapeutic and Preferred Concentrations
TGF- β and BMP-Receptor Agonists

<u>Compound</u>	<u>Therapeutic Concentrations (nanomolar)</u>	<u>Most Preferred Concentrations (nanomolar)</u>
TGF- β 1	0.05-500	0.5-100
TGF- β 2	0.05-500	0.5-100
BMP-2	0.1-2000	1-200
BMP-4	0.1-2000	1-200
BMP-7 (OP-1)	0.1-2000	1-200

5 5. Cyclooxygenase-2 (Cox-2) Inhibitors

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory agents, but have not been specifically developed or therapeutically employed as chondroprotective agents. The direct molecular target for an NSAID drug is the first enzyme in the prostaglandin synthetic pathway, referred to either as prostaglandin endoperoxide synthase or fatty acid cyclooxygenase. Two related forms of cyclooxygenase, termed cyclooxygenase-1 or type 1 (COX-1) and cyclooxygenase-2 (COX-2) have been characterized. These isozymes are also known as Prostaglandin G/ H Synthase (PGHS)-1 and PGHS-2. Both enzymes catalyze the rate-limiting step in the formation of prostanoids that is the conversion of arachidonic acid to prostaglandin H₂. COX-1 is present in platelets and endothelial cells and exhibits constitutive activity. In contrast, COX-2 has been identified in endothelial cells, macrophages, fibroblasts and other cells in the joint and its expression is induced by pro-inflammatory cytokines, such as IL-1 and TNF- α .

Within the inflamed joint, COX-2 expression is upregulated and it has been shown that large increases in activity of COX-2 occur concomitant with its upregulation, leading to increased synthesis of prostaglandins which are present in the synovial fluid of patients suffering from inflammatory arthropathies. Cellular sources of prostaglandins (PGs) in the joint include activated chondrocytes, type A and B synoviocytes and infiltrating macrophages. Cellular functions important in cartilage metabolism modulated by PGs include gene expression, extracellular matrix synthesis and proliferation. Because COX-2 is expressed in inflamed joint tissue or after exposure to mediators of inflammation (e.g., as a result of injury or surgical

trauma), the use of a COX-2 inhibitor is expected to provide both anti-inflammatory and cartilage protective activity.

Cartilage destruction in inflammatory arthropathies can be triggered as a consequence of joint injury and as a result of arthroscopic surgical procedures. Chondrocytes are the only cell type in articular cartilage and are known to participate in the breakdown of their own matrix through release of endogenous inflammatory mediators, including PGs. Studies have shown that COX-2 gene expression, protein synthesis, and PG release in normal human articular chondrocytes is rapidly induced by cytokines, including IL-1, TNF- α and IL-6. Levels of mRNA are detected as early as 2 hours after cytokine induction, reach high levels at 6 hours and show a remarkably long duration of expression for at least 72 hours. Similarly, cell culture studies of IL-1 α and TNF- α activation of human synoviocytes have shown large increases in expression of COX-2 and production of prostaglandin E2 (PGE2). Treatment with a variety of NSAIDs, such as ketoprofen, abolishes the induced PGE2 response. In a chondrocyte cell culture system, the specific COX-2 inhibitor compound NS-398 prevented the increase in PGE2 production induced by the cytokines while COX-1 levels remained stable (Morisset, S., 1998, J. Rheumatol. 25:1146-53). Thus, it can be deduced that blocking PG production by activated chondrocytes which is associated with expression of COX-2 can provide a chondroprotective effect.

NSAIDs are commonly used in the treatment of patients with osteoarthritis or rheumatoid arthritis, but their effects on articular cartilage metabolism in the context of these arthritic diseases remains a subject of debate. For instance, the clinical treatment of osteoarthritis and rheumatoid arthritis with NSAIDs is successful in reducing inflammation. However, it is thought that some NSAIDs which are not selective for COX-2, primarily salicylates and indomethacin, accelerate osteoarthritic cartilage destruction by impairing proteoglycan synthesis by chondrocytes, whereas other NSAIDs are thought to have a somewhat chondroprotective effect by stimulating cartilage repair. Most studies have shown that NSAIDs have little or no effect on cartilage. Due to the current lack of use of this class of drugs in the treatment of synovitis and cartilage destruction following traumatic joint injury and surgical trauma, the unique properties of each NSAID on the pathophysiological mechanisms that contribute to cartilage destruction will need to be established.

Since the two COX isozymes are pharmacologically distinct, isozyme-specific (selective) cyclooxygenase inhibitors that are useful for anti-inflammatory therapy have been developed and some of these same COX-2 inhibitors have been tested in

models of joint inflammation. However, the effects *in vitro* of the COX-2 inhibitors on the synthesis and degradation of cartilage proteoglycans, as well as synovial production of IL-1, IL-6, IL-8, and prostanoids, indicate that certain NSAIDs may vary considerably in their effects *in vivo* on cartilage and synovial production of interleukins and eicosanoids, such that the integrated effects of these parameters may influence the outcome of COX-2 inhibitors on cartilage integrity. For example, some NSAIDs can accelerate joint damage in osteoarthritis by enhancing the production of pro-inflammatory cytokines or inhibiting cartilage proteoglycan synthesis. However, despite the possible variance in clinical effect among COX-2 specific inhibitors, inhibition of COX-2 typically results in a reduction of synovitis and an expected decrease in the risk of cartilage damage.

A variety of biochemical and cellular and animal assays have been developed to assess the relative selectivity of inhibitors for the COX-1 and COX-2 isoforms. In general, a criteria for defining selectivity is the ratio of the COX-1/COX-2 inhibitory constants (or COX-2/COX-1) obtained for a given biochemical or cellular assay system. The selectivity ratio accounts for different absolute IC_{50} values for inhibition of enzymatic activity that are obtained between microsomal and cellular assay systems (e.g., platelet and macrophage cell lines stably expressing recombinant human COX isozymes). Furthermore, inhibition of COX-2 mimics the inhibitory effects triggered by chondroprotective (inhibitory) cytokines, such as IL-4, which down-regulate intracellular COX-2 synthesis. Comparison of the selectivity of more than 45 NSAIDs and selective COX-2 inhibitors (1997, Can. J. Physiol. Pharmacol. 75:1088-95) showed the following rank-ordered relative selectivity for COX-2 vs. COX-1: DuP 697 > SC-58451 = celecoxib > nimesulide = meloxicam = piroxicam = NS-398 = RS-57067 > SC-57666 > SC-58125 > flosulide > etodolac > L-745,337 > DFU-T-614, with IC_{50} values ranging from 7 nM to 17 μ M.

From the molecular and cellular mechanism of action defined for selective COX-2 inhibitors, such as celecoxib and rofecoxib, as well as from animal studies, these compounds are expected to exhibit chondroprotective action when applied perioperatively in an irrigation solution or in an injection directly to a joint. In particular, COX-2 inhibitors are expected to be effective drugs delivered in an irrigation solution during an arthroscopic surgical procedure or by direct injection into a joint prior to, during or after a surgical procedure or other injury to the joint.

Examples of COX-2 inhibitors suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is

therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 22

5

Therapeutic and Preferred Concentrations
of Cyclooxygenase-2 Inhibitors

<u>Compounds</u>	<u>Therapeutic Preferred Concentrations (nM)</u>	<u>Most Preferred Concentrations (nM)</u>
rofecoxib (MK 966)	0.3-30,000	30-3,000
SC-58451	0.3-30,000	30-3,000
celecoxib (SC-58125)	0.3-30,000	30-3,000
meloxicam	0.5-50,000	50-5,000
nimesulide	0.5-50,000	50-5,000
diclofenac	0.3-30,000	30-3,000
NS-398	0.3-30,000	30-3,000
L-745,337	0.2-100,000	20-10,000
RS57067	0.2-100,000	20-10,000
SC-57666	0.2-100,000	20-10,000
flosulide	0.2-100,000	20-10,000

6. MAP Kinase Inhibitors

The mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and function in transducing signals from the cell surface to the nucleus. The MAP kinase cascade is one of the major intracellular signalling pathways that transmit signals from growth factors, hormones and inflammatory cytokines to intermediate early genes. In combination with other signalling pathways, these activated mitogen-activated protein-kinases (MAPKs) differentially alter the phosphorylation state and activity of transcription factors, and ultimately regulate cell proliferation, differentiation and cellular response to environmental stress. For example, a member of the MAPK family (p38) mediates the major biochemical signal transduction pathways from the potent pro-inflammatory cytokines, IL-1 and TNF- α , leading to induction of cyclooxygenase-2 (COX-2) in stimulated macrophages, through cis-acting factors involved in the transcriptional regulation of the COX-2 gene.

The members of the MAP kinase class of agents are composed of at least three families that are known to differ in sequence, size of the activation loop, activation by extracellular stimuli and participation in distinct signal transduction pathways. Prominent members among this family of MAP kinases include the extracellular signal-regulated kinases (ERKs), ERK1 and ERK2 (p44MAPK and p42MAPK, respectively); stress-activated protein kinase 1 (SAPK1) family which is also referred to as the JNK or jun N-terminal kinase family; and the p38 MAP kinase family which is also known as stress-activated kinase 2/3 (SAPK-2/3). The p38 kinases are activated by stresses, most notably pro-inflammatory cytokines. Within the p38 family, there are at least four distinct homologs (isotypes or isoenzymes) which standard nomenclature refers to either as SAPK2a, SAPK2b, SAPK2d, SAPK3, or p38 α , β , δ (SAPK4) and γ , respectively. The inhibitors of MAP kinases useful for the practice of this invention may interact with any one or combination of the above MAP kinases. For specific MAP kinase inhibitors, the inhibitory constants characterized through assays of purified *in vitro* enzymes and in cellular assays may vary over a wide range of concentrations and demonstrate utility in this application. Activation of p38 MAP kinase is mediated by dual phosphorylation of threonine and tyrosine residues. Both TNF- α and IL-1 treatment of cells has been shown to rapidly (within 5 min) increase phosphorylation and activate p38 MAP kinase.

Previous work has shown that small-molecule inhibitors can specifically inhibit p38 MAP kinase (Lee, J. et al., *Nature* **372**: 739-746 (1994)) and produce anti-inflammatory effects at the biochemical level and in various animal models. Cuenda and coworkers (Cuenda, A. et al., *FEBS Lett.* **364**: 229 (1995)) showed that the compound, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] inhibited p38 *in vitro* (IC₅₀ = 0.6 μ M), suppressed the activation of MAPK activating protein kinase-2 and prevented the phosphorylation of heat shock protein (hsp) 27 in response to IL-1 and cellular stresses *in vivo*. The kinase selectivity of SB203580 inhibitory action for p38 was demonstrated by its failure or at best weak inhibition of at least 15 other protein kinases *in vitro*, including members of the PKC, PKA, src and receptor tyrosine kinase families (Lee, J., *Pharmacol. Ther.* **82**: 389-397 (1999)). In cellular studies, pre-incubation with SB 203580 has been shown to block the IL-1 and TNF- α induced phosphorylation of the enzyme and subsequent IL-8 production. This supports the preemptive effect of delivering the inhibitors during the surgical procedure.

The role of p38 mitogen-activated protein kinase (MAPK) in biochemical inflammatory responses resulting in destruction of cartilage has been studied using

SB203580, which specifically inhibits the enzyme. Actions of IL-1 that are selectively controlled by p38 MAPK are the regulation of prostaglandin H synthase-2 (COX-2), metalloproteinases, and IL-6 (Ridley, S et al., 1997, J. Immunol. 158:3165-73). In human fibroblasts and vascular endothelial cells, SB203580 inhibited (IC₅₀= 0.5 μ M) IL-1-induced phosphorylation of hsp 27 (an indicator of p38 MAPK activity) in fibroblasts without affecting the other known IL-1-activated protein kinase pathways (p42/p44 MAPK, p54 MAPK/c-Jun N-terminal kinase). In addition, SB203580 significantly inhibited IL-1-stimulated IL-6 (30 to 50% at 1 μ M) but not IL-8 production from human fibroblasts and endothelial cells.

Importantly, SB203580 strongly inhibited IL-1-stimulated prostaglandin production by fibroblasts and human endothelial cells. This was associated with the inhibition of the induction of COX-2 protein and mRNA. PGE₂ contributes to increased expression of matrix metalloproteinases that are important mediators of cartilage degradation. Both synovial fibroblasts and chondrocytes express the COX-2 gene at high levels upon activation by cytokines and extracellular stimuli. The MAPK inhibitor provides chondroprotective activity due to its inhibitory activity on MAP kinases expressed in these and other cell types.

MAPK inhibitors are expected to be effective as cartilage protective agents when applied locally to tissues of the joint in a variety of inflammatory or pathophysiological conditions. SB 203580 has been characterized in several pharmacological models *in vivo* and demonstrated to have activity under long term, oral dosing. SB203580 was found to inhibit the stimulation of collagenase-1 and stromelysin-1 production by IL-1 without affecting synthesis of TIMP-1. Furthermore, SB203580 prevented an increase in IL-1-stimulated collagenase-1 and stromelysin-1 mRNA. In a model of cartilage breakdown, short-term IL-1-stimulated proteoglycan resorption and inhibition of proteoglycan synthesis were unaffected by SB 203580, while longer term collagen breakdown was prevented. In addition, SB203580 was found to be effective in inhibiting IL-1-induced nitric oxide production in bovine articular cartilage explants and chondrocytes (Badger 1998). These *in vitro* observations provide a basis for cartilage protective activity of the MAP kinase inhibitor administered directly and locally to these tissues in the joint.

p38 MAP kinase is involved in TNF-induced cytokine expression, and drugs which function as inhibitors of p38 MAP kinase activity block the production of pro-inflammatory cytokines (Beyaert, R. et al., *EMBO J.* 15:1914-23 (1996)). TNF- α treatment of cells activated the p38 MAPK pathway as shown by increased

phosphorylation of p38 MAPK itself and activation of its substrate proteins. Pretreatment of cells with SB203580 completely blocked TNF- α induced activation of MAPK activating protein kinase-2 and hsp27 phosphorylation. Under the same conditions, SB203580 also completely inhibited TNF- α induced synthesis of IL-6 and expression of a reporter gene that was driven by a minimal promoter containing two NF- κ B elements. Thus, these studies and related studies on other p38 inhibitors show that the action of inhibitors, such as SB203580 and FR133605, on p38 MAPK interfere selectively with TNF- α - and IL-1-induced activation of various proteins linked to the cartilage degradation. Thus, the selective inhibition of the MAP kinase signalling pathways of these key pro-inflammatory cytokines by inhibition of a kinase downstream of the receptor indicate that MAP kinase inhibitors may provide a chondroprotective effect.

SB 203580 has been evaluated in several animal models of cytokine inhibition and inflammatory disease. It was demonstrated to be a potent inhibitor of inflammatory cytokine production *in vivo* in both mice and rats with IC₅₀ values of 15 to 25 mg/kg. SB 203580 possessed therapeutic activity in collagen-induced arthritis in DBA/LACJ mice with a dose of 50 mg/kg resulting in significant inhibition of paw inflammation. Antiarthritic activity was also observed in adjuvant-induced arthritis in the Lewis rat when SB203580 was administered p.o. at 30 and 60 mg/kg/day. Additional evidence was obtained for beneficial effects on bone resorption with an IC₅₀ of 0.6 μ M.

In summary, a variety of biochemical, cellular and animal studies show that p38 MAPK plays an important role in the regulation of responses to IL-1 and TNF- α and that it is involved in the regulation of mRNA levels of some inflammatory-responsive genes, such as COX-2. Inhibitors of p38 block the production of pro-inflammatory cytokines and inhibit the production of MMPs, and have been demonstrated to inhibit collagen breakdown in cartilage explants.

The use of MAPK inhibitor to block the actions of key pro-inflammatory cytokines, such as IL-1 and TNF- α , will have beneficial effects on many cell types in the joint, including synovial fibroblasts, macrophages and chondrocytes, thus inhibiting subsequent pathological effects such as infiltration of inflammatory cells into the joint, synovial hyperplasia, synovial cell activation, and cartilage breakdown. Thus, a MAPK inhibitor should block the propagation of the inflammatory response by the aforementioned cytokines, and thereby interrupt the disease process.

Examples of MAPK inhibitors suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation) the

optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

5

Table 23

Therapeutic and Preferred Concentrations of MAP Kinase Inhibitors

<u>Compounds</u>	<u>Therapeutic Concentrations (nanomolar)</u>	<u>Preferred Concentrations (nanomolar)</u>
SB 203580	0.5-50,000	50-5,000
SB 203580 iodo	0.5-50,000	50-5,000
SB 202190	0.2-20,000	20-2,000
SB 242235	0.2-10,000	20-1,000
SB 220025	0.2-10,000	20-1,000
RWJ 67657	0.3-30,000	30-3,000
RWJ 68354	0.9-90,000	90-9,000
FR133605	1-100,000	10-10,000
L-167307	0.5-50,000	50-5,000
PD 98059	0.1-10,000	10-1000
PD 169316	1-100,000	10-10,000

7. Inhibitors Of Matrix Metalloproteinases

- 10 Destruction of articular cartilage is a common feature in joint diseases such as osteoarthritis and rheumatoid arthritis, but also occurs following injury to the joint. Pathophysiologically, a structural breakdown of proteoglycans and collagen is observed, which impairs the biomechanical properties of cartilage. The maintenance of a normal, healthy extracellular matrix reflects a balance between the rate of
- 15 biosynthesis and incorporation of matrix components, and the rate of their degradation and subsequent loss from the cartilage into the synovial fluid. A variety of proteases have the potential to cleave cartilage and are involved in the degradation process, most notably the matrix metalloproteinases.

Matrix metalloproteinases (MMPs), or matrixins, are a family of at least 15 zinc endopeptidases that function extracellularly and play a key role in pathological degradation of tissue. Current nomenclature and alternative names for members of the MMP are provided in Table 23. Most MMPs are highly regulated and most are not constitutively expressed in normal tissues. However, pro-inflammatory cytokines, such as IL-1 and TNF- α , initiate transcription and expression. An imbalance created by upregulation and activation of tissue-degrading MMPs is a primary causative factor in the cartilage breakdown process during chronic inflammatory diseases and sustained synovial inflammatory responses subsequent to joint injury. Cartilage matrix metabolism has been studied in patients with either a meniscal injury or anterior cruciate ligament rupture in the knee. It was shown that concentrations of stromelysin-1 (MMP-3), collagenase, tissue inhibitor of metalloproteinases (TIMP-1), and proteoglycan fragments increased in human knee synovial fluid after traumatic knee injury. Temporally, these values increased immediately over reference levels and remained significantly elevated (10-fold increase) over a period of one year. These changes likely drive the increase in the concentration of proteoglycan fragments that are observed in synovial fluid after knee ligament injury.

Table 24

<u>Matrix Metalloproteinases</u>			
<u>MMP</u>	<u>Alternative Names</u>	<u>EC Number</u>	<u>Substrates</u>
MMP-1	Collagenase Fibroblast Collagenase Interstitial Collagenase	EC3.4.24.7	Collagens (I, II, III, VII, and X); Gelatin; aggrecan; hyaluronidase-treated versican; proteoglycan link protein; large tenascin-C; α_1 - antitrypsin/ α_1 - proteinase inhibitor (α_1 - AT); α_1 antichymotrypsin (α_1 - ACHYM); α_2 M; rat α_1 M; pregnancy zone protein; rat α_1 I ₃ (α_1 - inhibitor 3); ovostatin; entactin; MBP; GST-TNF/TNF peptide; L-selection; IL-1 β ; serum amyloid A; IGF-BP5; IGF-BP3; MMP-2; MMP-13
MMP-2	72-kDa Gelatinase Gelatinase A Type IV Collagenase Neutrophil Gelatinase	EC3.4.24.24	Collagens (I, IV, V, VI, X, XI, and XIV); Gelatin; elastin; fibronectin; laminin-1, laminin-5; gelatin-3; aggrecan; decorin; hyaluronidase-treated versican; proteoglycan link protein; osteonectin; MBP; GST-TNF/TNF peptide; IL-1 β ; A β ₁₋₄₀ ; A β ₁₀₋₂₀ ; APP ₆₉₅ ; α_1 - AT; prollysyl oxidase fusion protein; IGF-BP5; IGF-BP3; FGF R1; MMP-1; MMP-9; MMP-13
MMP-3	Stromelysin-1 Transin	EC3.4.24.17	Collagens (III, IV, V, IX); Gelatin; aggrecan; versican and hyaluronidase-treated versican; perlecan; decorin; proteoglycan link protein; large tenascin-C; fibronectin; laminin; entactin; osteonectin; elastin; casein; α_1 - ACHYM; antithrombin-III; α_2 M; ovostatin; Substance P; MBP; GST-TNF/TNF peptide; IL-1 β ; serum amyloid A; IGF-BP3; fibrinogen and cross-linked fibrin; plasminogen; MMP-1 "superactivation", MMP-2/TIMP-2 complex; MMP-7; MMP-8; MMP-9; MMP-13

MMP-7	Matrilysin PUMP	EC3.4.24.23	Collagen IV and X; Gelatin; aggrecan; decorin; proteoglycan link protein; fibronectin and laminin; insoluble fibronectin fibrils; enactin; large and small tenascin-C; osteonectin; β 4 integrin; elastin; casein; transferrin; MBP; α_1 -AT; GST-TNF/TNF peptide; plasminogen; MMP-1; MMP-2; MMP-9; MMP-9/TIMP-1
MMP-8	Neutrophil Collagenase Collagenase I	EC3.4.24.34	Collagens (I, II, III, V, VII and X); Gelatin; aggrecan; α_1 -AT; α_1 -ACHYM; α_2 -antiplasmin; fibronectin
MMP-9	92 kDa Gelatinase Gelatinase B	EC3.4.24.35	Collagens (IV, V, VII, X and XIV); Gelatin; elastin; galectin-3; aggrecan; hyaluronidase-treated versican; proteoglycan link protein; fibronectin; entactin; osteonectin; α_1 -AT; MBP; GST-TNF/TNF peptide; IL-1 β ; A β_{1-40} ; plasminogen
MMP-10	Stromelysin-2	EC3.4.24.22	Collagens (III, IV and V); Gelatin; casein; aggrecan; elastin; proteoglycan link protein; MMP-1; MMP-8
MMP-11	Stromelysin-3		Human enzyme: α_1 -AT; α_2 M; casein, laminin, fibronectin, gelatin, collagen IV and carboxymethylated transferrin
MMP-12	Macrophage Metalloelastase		Collagen IV; Gelatin; elastin and κ -elastin; casein; α_1 -AT; fibronectin; vitronectin; laminin; enactin; proteoglycan monomer; GST-TNF; MBP; fibrinogen; fibrin; plasminogen
MMP-13	Collagenase-3		Collagens (I, II and III, IV, IX, X and XIV); Gelatin, α_1 -ACHYM and plasminogen activator inhibitor 2; aggrecan; perlecan; large tenascin-C, fibronectin; osteonectin; MMP-9
MMP-14	MT-MMP-1		Collagen (I, II and III); Gelatin, casein, κ -elastin, fibronectin, laminin, vitronectin and

		proteoglycans; large tenascin-C, enactin; α_1 -AT, α_2 M; GST-TNF; MMP-2; MMP-13
MMP-15	MT-MMP-2	Fibronectin, large tenascin-C, entactin, laminin, aggrecan, perlecan; GST-TNF; MMP-2

The MMP family of enzymes has been shown to be secreted from human chondrocytes and by cells of the synovium, such as synovial fibroblasts. Furthermore, using *in situ* hybridization, it was shown that human synovium synthesizes both stromelysin-1 and collagenase. Stromelysin-1 (MMP-3) is capable of degrading all of the components of the cartilage matrix. There is evidence that chondrocytes contribute to cartilage degradation by the release of the matrix-degrading enzyme, collagenase-3. Upon activation by pro-inflammatory cytokines, MMPs are secreted from cells in a latent form, are activated extracellularly, and are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The balance between the activities of MMPs and TIMPs is thought to be important for the maintenance of an intact cartilage matrix. Under pathological conditions such as osteoarthritis and rheumatoid arthritis, several studies have shown elevated amounts of MMPs, resulting in an imbalance between MMPs and TIMPs that is considered to account for the observed cartilage destruction.

The MMPs are regulated by cytokines, such as interleukin-1 (IL-1), and growth factors that act on chondrocytes and synoviocytes to enhance their protease production. Other pro-inflammatory cytokines, such as IL-6, IL-8 and TNF- α , also upregulate the production of matrix-degrading enzymes. This leads to cartilage destruction, which is usually assessed as the loss of sulfated glycosaminoglycans (GAGs) and the cleavage of collagen. IL-1, which is present in the joint fluid of patients with arthritic diseases, stimulates chondrocytes to synthesize elevated amounts of enzymes such as stromelysin, fibroblast and neutrophil collagenase, and plasminogen activator. In addition, IL-1 inhibits synthesis of plasminogen activator inhibitor-1 and TIMP, and also inhibits synthesis of matrix constituents such as collagen. The imbalance between the levels of inhibitors and enzymes leads to an increase in the amount of active proteases and, combined with a suppression of matrix biosynthesis, results in cartilage degradation.

Using cartilage slices as an *in vitro* model, it has been shown that collagenase inhibitors can inhibit either the IL-1 or IL-8 stimulated invasion of articular cartilage by rheumatoid synovial fibroblasts (RSF). The collagenase inhibitors, 1,10-o-

phenanthroline and phosphoramidon, substantially inhibited the concentration-dependent penetration of cartilage by RSF cells at concentrations of 10-150 μ M. The selective effect of cytokines on the secretion of proteinases demonstrates that synovial fibroblast-like cell-mediated articular degradation is a highly regulated process. Thus, the ability to inhibit protease activity and associated matrix degradation locally within the joint is expected to inhibit the cartilage destruction process. The action of the inhibitors in the limited *in vitro* system suggests that therapeutic intervention using local delivery of synthetic MMP inhibitors with appropriate pharmacokinetics will be effective as chondroprotective agents.

Examples of MMP inhibitors suitable for the present invention include U-24522 ((R,S)-N-[2-[2-(hydroxylamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide), peptides such as MMP Inhibitor I and MMP-3 Inhibitor, and larger proteins such as TIMP-1 or fragments thereof, and are listed in the Table below: For all modes of local delivery (i.e., injection, infusion and irrigation) the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 25

Therapeutic and Preferred Concentrations of Matrix Metalloproteinases (MMPs)
Inhibitors

<u>Compounds</u>	<u>Therapeutic Concentrations (nanomolar)</u>	<u>Most Preferred Concentrations (nanomolar)</u>
U-24522	0.2-2,000	20-200
minocycline	30-500,000	300-3,000
MMP Inhibitor I	0.3-3,000	3-600
4-Abz-Gly-Pro-D-Leu-D-Ala-NHOH		
MMP-3 Inhibitor	0.5-5,000	5-500
Ac-Arg-Cys-Gly-Val-Pro-Asp-NH ₂		
rhuman TIMP1	0.5-5,000	5-500
rhuman TIMP2	0.3-3,000	3-600
phosphoramidon	1,000-500,000	5,000-100,000

8. Inhibitors Of Nuclear Factor Kappa B (NFkB)

Pro-inflammatory and cartilage-destructive cellular pathways are regulated by extracellular and intracellular signalling mechanisms that are targets for novel therapeutic local drug delivery. The complete molecular signaling mechanisms utilized by the pro-inflammatory cytokine interleukin-1 (IL-1) to activate the transcription factor, nuclear factor kappaB (NFkB), are poorly defined. Nevertheless, a key molecule that is involved in intracellular signalling at the level of gene transcription is the pro-inflammatory transcription factor, (NFkB). NFkB activity is mediated by a family of transcription factor subunits that bind to DNA either in the form of homodimers or heterodimers. These subunits are typically present within the cytoplasm of cells in an inactive form due to the binding of the inhibitory subunit called IkB. Activation of IL-1 receptors, and other extracellular signals, induce degradation of IkB and concomitant dissociation of NFkB from the inhibitors, followed by translocation to the nucleus. NFkB, was found to be involved in IL-1 induced expression and was capable of increasing pro-inflammatory COX-2 protein expression in RA synovial fibroblasts.

The identification of NFkB as a key molecular target is based upon its role as a common downstream signaling element regulating gene expression of several critical inflammatory mediators linked to joint inflammation and cartilage-destructive pathways. The response of many genes (COX-2, collagenase, IL-6, IL-8) are governed by promoters which contain both NFkB promoter elements. Activation of NFkB mediates the induction of many proteins central to the inflammatory process, such as cytokines, cell-adhesion molecules, metalloproteinases and other proteins that participate in the production of prostaglandins and leukotrienes (COX-2) in synoviocytes. Thus, this transcription factor represents a physiologically significant target in therapies directed to the injury responses of human synovial fibroblasts, human articular chondrocytes, as well as other cells in the joint.

Specifically, it has been shown that exposure of human rheumatoid synovial fibroblasts (RSF) to interleukin 1beta (IL-1beta) results in the coordinate up-regulation of 85-kD phospholipase A2 (PLA2) and inducible cyclooxygenase (COX-2). Together, these two enzymes promote the subsequent biosynthesis of PGE₂, a primary inflammatory mediator in the joint. Oligonucleotide decoys and antisense were used to demonstrate the participation of the (NFkB), in the regulation of the prostanoid-metabolizing enzymes. Antagonizing NFkB mRNA using anti-sense oligonucleotide resulted in decreased binding to the COX gene promoter.

Hymenialdisine, a marine natural product, has recently been characterized as an inhibitor of NFκB activation and exposure of IL-1-stimulated RSF-inhibited PGE2 production in a concentration-dependent manner ($IC_{50} = 1 \mu M$). The specificity of the molecular target was shown through use of an analog, aldisine, and the protein kinase C inhibitor, RO 32-0432, which were inactive. Direct action of hymenialdisine on IL-1-induced NFκB activation was demonstrated by a significant reduction (approximately 80%) in NFκB binding to the classical κB consensus motif and inhibition of stimulated p65 migration from the cytosol of treated cells. As expected for an inhibitor of NFκB transcriptional regulation, hymenialdisine-treated RSF did not transcribe the mRNAs for either COX-2 or PLA2 in response to IL-1. Consequently, reduced protein levels for these enzymes and reductions in the ability to produce PGE2 were observed. Furthermore, IL-1-stimulated interleukin-8 (IL-8) production, which is known to be an NFκB-regulated event, was also inhibited by hymenialdisine, whereas IL-1-induced production of vascular endothelial growth factor, a non-NFκB-regulated gene, was not affected by exposure to hymenialdisine. Thus, hymenialdisine inhibits IL-1-stimulated synovial fibroblast formation of PGE2 through its inhibitory effect on NFκB activation. This provides a basis to define its use as a novel inhibitor to block the role of NFκB in joint inflammation and cartilage destruction.

Examples of NFκB inhibitors suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation), the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective.

Table 26**Therapeutic and Preferred Concentrations of Inhibitors of NFkB**

<u>Compounds</u>	<u>Therapeutic Concentrations (nanomolar)</u>	<u>Most Preferred Concentrations (nanomolar)</u>
Caffeic acid phenylethyl ester (CAPE)	1-100,000	50-20,000
DM-CAPE	0.5-50,000	50-5,000
SN-50 peptide	0.1-100,000	100-20,000
hymenialdisine	1-100,000	100-10,000
pyrolidone dithiocarbamate	1-50,000	50-10,000

9. Nitric Oxide Synthase Inhibitors

Nitric oxide (NO) is a widespread intracellular and intercellular mediator involved in the pathophysiological mechanisms of some connective tissue diseases. NO is formed from L-arginine by a family of enzymes, the NO synthases, which are localized intracellularly. Three isoforms of NO synthase have been cloned and sequenced. Endothelial cell NO synthase (ecNOS) and brain NO synthase (bNOS) are constitutively active. A distinct isoform of NO synthase, inducible NOS (iNOS), is found in many cell types, including chondrocytes. It is absent under basal conditions, but is upregulated in response to pro-inflammatory mediators such as IL-1 β and TNF- α . Recent findings show that IL-1 is a very potent stimulator of chondrocyte NO synthesis and that IL-1 acts through its ability to upregulate the level of the iNOS. Within the joint, chondrocytes are the major source of NO and chondrocytic iNOS induced by pro-inflammatory cytokines is considered to mediate many effects of IL-1 in inflammatory arthropathies.

Drugs that specifically inhibit chondrocyte inducible NO synthase (iNOS) may have a therapeutic role in the prevention of chondrodestruction that occurs due to joint injury (e.g., surgical procedures involving the joint). Evidence supporting such a beneficial therapeutic effect is based upon a substantial number of studies which have evaluated a variety of iNOS inhibitors for their ability to inhibit inducible NO synthase activity in cultured chondrocytes and explants of cartilage from patients with osteoarthritis. A class of compounds, termed S-substituted isothioureas, have been characterized as potent inhibitors of NO biosynthesis in cartilage. S-methyl isothiourea and S-(aminoethyl) isothiourea were 2-4 times more potent than N^G-monomethyl-L-arginine, 5-10 times more potent than aminoguanidine and over 300 times more potent than N^w-nitro-L-arginine and N^w-nitro-L-arginine methyl ester.

These isothiourea compounds provide a potent and relatively specific class of inhibitors of iNOS in cartilage and thus are suitable for local delivery in the current invention (Jang, D., 1996, *Eur. J. Pharmacol.* 312: 341-347).

The cartilage protective therapeutic potential of NO synthase inhibitors has also been assessed using *in vitro* systems such as isolated chondrocytes to define effects on the cartilage matrix. Inhibition of endogenous NO production by N^G - monomethyl-L-arginine (L-NMMA), an established NO synthase inhibitor, leads to the suppression of gelatinase, collagenase, and stromelysin production by IL-1 β -stimulated chondrocytes. Inhibition of NO production also partially reduces the increase in the lactate production that occurs from the exposure of chondrocytes to IL-1 β . Treatment of cartilage fragments with L-NMMA partially reverses the IL-1 β inhibitory effect of glycosaminoglycan synthesis, inhibits IL-1 β -stimulated MMP activities, and increases IL-1 receptor antagonist (IL-1ra) production. NO can also modulate proteoglycan synthesis indirectly by decreasing the production of TGF- β 1 by chondrocytes exposed to IL-1 β . It prevents autocrine-stimulated increases in TGF- β 1, thus diminishing the anabolic effects of this cytokine in chondrocytes.

A study has compared the potency of new aminoguanidine, S-methylisothiourea (SMT), S-aminoethylisothiourea (AETU), L-NMMA and N-nitro-L-arginine methyl ester (L-NAME) NOS inhibitors on the inhibitory effect of recombinant human IL-1 responses on proteoglycan synthesis and NO production. Different culture systems have been shown to respond in a concentration dependent manner to IL-1 β challenge with a large increase in NO production and a marked suppression of proteoglycan synthesis. The above NOS inhibitors (at 1 to 1000 μ M) inhibited NO production by cartilage cells treated with IL-1 β and had marked effects on restoring proteoglycan synthesis in chondrocytes. Therefore, if NO production can be blocked using a therapeutically effective concentration and dose, then IL-1 β inhibition of proteoglycan synthesis will be prevented.

NO synthase is expressed in cartilage obtained from the joint of patients with arthritic disease. In patients presenting either rheumatoid arthritis or osteoarthritis, increased levels of nitrite have been observed in the synovial fluid and it has been shown that a significant source of NO production in these patients is derived from articular cartilage. Furthermore, it has been found that sustained systemic delivery of L-NIL, a potent inhibitor of iNOS, reduces the progression of experimental OA in dogs (induced by sectioning of the ACL) and causes a substantial decrease in IL-1 β , PGE₂, NO and MMP production. These findings suggest that NO is a potent

regulator of the effects of IL-1 β and contributes to the pathophysiology of joint diseases.

Thus, these *in vitro* and *in vivo* results indicate that specific inhibitors of NO synthases are potential novel drugs for the clinical treatment of synovial inflammation and can provide chondroprotective effects when delivered locally in combination with one or more drugs chosen from the anti-inflammatory, cartilage-protective, and anti-pain classes to treat a surgically treated joint or other injured joint.

Examples of NO synthase inhibitors suitable for the present invention are listed below. For all modes of local delivery (i.e., injection, infusion and irrigation), the optimal dose and/or concentration of each suitable agent is that which is therapeutically effective. As an example, for each of the listed agents, the preferred and most preferred concentrations of an irrigation solution containing the listed agent are provided, such concentrations expected to be therapeutically effective. In one embodiment, the preferred NO synthase inhibitors for inclusion in the solutions of the invention is 1400 W ((N-3-(aminomethyl)benzyl)acetamidine), a selective, slow, tight binding inhibitor of iNOS, diphenyleneiodinium and 1,3-PBIT.

Table 27

**Therapeutic and Preferred Concentrations of
Nitric Oxide Synthase Inhibitors**

<u>Compounds</u>	<u>Therapeutic Concentrations (μM)</u>	<u>Most Preferred Concentrations (μM)</u>
N ^G -monomethyl-L-arginine	50-50,000	3,000
1400 W	0.1-1,000	1-20
diphenyleneiodinium	0.1-1,000	1-100
S-methyl isothiurea	1-1,000	10-100
S-(aminoethyl) isothiurea	1-1,000	10-100
L-N ⁶ -(1-iminoethyl)lysine	1-1,000	10-100
1,3-PBITU	0.5-500	5-100
2-ethyl-2-thiopseudourea	2-20,000	20-2,000

10. Cell Adhesion Molecules

10a Integrin Agonists and Antagonists

Integrins are heterodimer receptors located on the plasma membrane that contain α and β subunits that bind ligands which are extracellular matrix (ECM) components or may be other large proteins, such as collagen, laminin, vitronectin, osteopontin (OPN) and fibronectin (FN). Degradation of the cartilage matrix is regulated by chondrocytes through mechanisms which depend upon the interaction of these cells with the ECM. Chondrocyte gene expression is, in part, controlled through cellular contacts involving the interaction of integrins with components of ECM in the environment surrounding the chondrocyte. Hence, integrins on chondrocytes are involved in control of cartilage homeostasis, and this family of receptors represents a class of therapeutic targets for preventing cartilage degradation.

Human chondrocytes express an array of integrin receptors composed of distinct α and β subunits, including $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha V\beta_1$ and lesser quantities of others. Of particular importance is the $\alpha V\beta_3$ integrin, which is known to bind OPN. The $\alpha V\beta_3$ complex-specific function blocking monoclonal antibody (mAb) LM609 acts as an agonist in a manner that is similar to the ligand, OPN. It attenuates the production of a number of proinflammatory and cartilage destructive mediators, such as IL-1, NO and PGE2. Thus, the agonistic mAb LM609 is thought to be suitable for use in the present invention.

In addition, two peptidomimetics, MK-383 (Merck) and RO 4483 (Hoffmann-LaRoche), have been studied in Phase II clinicals. Since these are both small molecules, they have a short half-life and high potency. However, these seem to also have less specificity, interacting with other closely related integrins. These peptidomimetics are also be suitable for use in the present invention.

Table 28
Therapeutic and Preferred Concentrations of
Integrins

<u>Class of Agent</u>	<u>Therapeutic Concentrations ($\mu\text{g/ml}$)</u>	<u>Preferred Concentrations ($\mu\text{g/ml}$)</u>
Integrins:		
$\alpha V\beta_3$ mAb LM 609	0.05-5,000	5-500
echistatin	0.1-10,000	100-1,000

11. Anti-chemotactic agents

Anti-chemotactic agents prevent the chemotaxis of inflammatory cells. Representative examples of anti-chemotactic targets at which these agents would act include, but are not limited to, F-Met-Leu-Phe receptors, IL-8 receptors, MCP-1
5 receptors, and MIP-1-I/RANTES receptors. Drugs within this class of agents are early in the development stage, but it is theorized that they may be suitable for use in the present invention.

12. Intracellular Signaling Inhibitors

12a. Protein Kinase Inhibitors

10 i. Protein Kinase C (PKC) Inhibitors

Protein kinase C (PKC) plays a crucial role in cell-surface signal transduction for a number of physiological processes. PKC isozymes can be activated as downstream targets resulting from initial activation of either G-protein coupled receptors (e.g., serotonin, bradykinin, etc.) or pro-inflammatory cytokine receptors.
15 Both of these receptor classes play important roles in mediating cartilage destruction.

Molecular cloning analysis has revealed that PKC exists as a large family consisting of at least 8 subspecies (isozymes). These isozymes differ substantially in structure and mechanism for linking receptor activation to changes in the proliferative response of specific cells. Expression of specific isozymes is found in
20 a wide variety of cell types, including: synoviocytes, chondrocytes, neutrophils, myeloid cells, and smooth muscle cells. Inhibitors of PKC are therefore likely to effect signaling pathways in several cell types unless the inhibitor shows isozyme specificity. Thus, inhibitors of PKC can be predicted to be effective in blocking the synoviocyte and chondrocyte activation and may also have an anti-inflammatory
25 effect in blocking neutrophil activation and subsequent attachment. Several inhibitors have been described and initial reports indicate an IC_{50} of 50 μM for calphostin C inhibitory activity. G-6203 (also known as Go 6976) is a new, potent PKC inhibitor with high selectivity for certain PKC isotypes with IC_{50} values in the 2-10 μM range. Concentrations of these and another drug, GF 109203X, also known
30 as Go 6850 or bisindoylmaleimide I (available from Warner-Lambert), that are believed to be suitable for use in the present invention are set forth below.

Table 29**Therapeutic and Preferred Concentrations of
Cartilage Destruction Inhibitory Agents**

<u>Class of Agent</u>	<u>Therapeutic Concentrations (Nanomolar)</u>	<u>Preferred Concentrations (Nanomolar)</u>
Protein Kinase C Inhibitors:		
calphostin C	0.5-50,000	100-5,000
GF 109203X	0.1-10,000	1-1,000
G-6203 (Go 6976)	0.1-10,000	1-1,000

ii. Protein Tyrosine Kinase Inhibitors

5 Although there is a tremendous diversity among the numerous members of the receptors tyrosine-kinase (RTK) family, the signaling mechanisms used by these receptors share many common features. Biochemical and molecular genetic studies have shown that binding of the ligand to the extracellular domain of the RTK rapidly activates the intrinsic tyrosine kinase catalytic activity of the intracellular domain (see

10 **FIGURE 5**). The increased activity results in tyrosine-specific phosphorylation of a number of intracellular substrates which contain a common sequence motif. Consequently, this causes activation of numerous "downstream" signaling molecules and a cascade of intracellular pathways that regulate phospholipid metabolism, arachidonate metabolism, protein phosphorylation (involving mechanisms other than

15 protein kinases), calcium mobilization and transcriptional activation (see **FIGURE 2**). Growth-factor-dependent tyrosine kinase activity of the RTK cytoplasmic domain is the primary mechanism for generation of intracellular signals that lead to cellular proliferation. Thus, inhibitors have the potential to block this signaling and thereby prevent synoviocyte and chondrocyte activation.

20 Any of several related tyrphostin compounds have potential as specific inhibitors of tyrosine kinase activity (IC_{50} s *in vitro* in the 0.5-1.0 μ M range), since they have little effect on other protein kinases and other signal transduction systems. To date, only a few of the many tyrphostin compounds are commercially available, and suitable concentrations for these agents as used in the present invention are set

25 forth below. In addition, staurosporine has been reported to demonstrate potent inhibitory effects against several protein tyrosine kinases of the src subfamily and a suitable concentration for this agent as used in the present invention also is set forth below.

Table 30
Therapeutic and Preferred Concentrations of
Inhibitory Agents

<u>Class of Agent</u>	Therapeutic Concentrations (Nanomolar)	Preferred Concentrations (Nanomolar)
Protein Kinase Inhibitors		
lavendustin A	10-100,000	100-10,000
tyrphostin	10-100,000	100-10,000
AG1296		
tyrphostin	10-100,000	100-10,000
AG1295		
staurosporine	1-100,000	10-1,000
PD 158780	0.1-10,000	10-500
PD 174265	0.1-10,000	10-500

5 12b. Modulators of Intracellular Protein Tyrosine Phosphatases.

Non-transmembrane protein tyrosine phosphatases (PTPases) containing src-homology₂ SH2 domains are known and nomenclature refers to them as SH-PTP1 and SH-PTP2. In addition, SH-PTP1 is also known as PTP1C, HCP or SHP. SH-PTP2 is also known as PTP1D or PTP2C. Similarly, SH-PTP1 is expressed at high levels in hematopoietic cells of all lineages and all stages of differentiation, and the SH-PTP1 gene has been identified as responsible for the motheaten (me) mouse phenotype and this provides a basis for predicting the effects of inhibitors that would block its interaction with its cellular substrates. Stimulation of neutrophils with chemotactic peptides is known to result in the activation of tyrosine kinases that mediate neutrophil responses (Cui, et al., 1994 J. Immunol.) and PTPase activity modulates agonist induced activity by reversing the effects of tyrosine kinases activated in the initial phases of cell stimulation. Agents that could stimulate PTPase activity could have potential therapeutic applications as anti-inflammatory mediators.

20 These same PTPases have also been shown to modulate the activity of certain RTKs. They appear to counter-balance the effect of activated receptor kinases and thus may represent important drug targets. *In vitro* experiments show that injection of PTPase blocks insulin stimulated phosphorylation of tyrosyl residues on endogenous proteins. Thus, activators of PTPase activity could serve to reverse activation of RTK-receptor action in restenosis, and are believed to be useful in the

solutions of the present invention. In addition, receptor-linked PTPases also function as extracellular ligands, similar to those of cell adhesion molecules. The functional consequences of the binding of a ligand to the extracellular domain have not yet been defined but it is reasonable to assume that binding would serve to modulate phosphatase activity within cells (Fashena and Zinn, 1995, *Current Biology*, 5, 1367-1369). Such actions could block adhesion mediated by other cell surface adhesion molecules (NCAM) and provide an anti-inflammatory effect. No drugs have been developed yet for these applications.

12c. Inhibitors of SH2 Domains (src Homology₂ Domains)

SH2 domains, originally identified in the src subfamily of protein tyrosine kinases (PTKs), are noncatalytic protein sequences and consist of about 100 amino acids conserved among a variety of signal transducing proteins (Cohen, et al., 1995). SH2 domains function as phosphotyrosine-binding modules and thereby mediate critical protein-protein associations in signal transduction pathways within cells (Pawson, *Nature*, 573-580, 1995). In particular, the role of SH2 domains has been clearly defined as critical for receptor tyrosine kinase (RTK) mediated signaling such as in the case of the platelet-derived growth factor (PDGF) receptor. Phosphotyrosine-containing sites on autophosphorylated RTKs serve as binding sites for SH2-proteins and thereby mediate the activation of biochemical signaling pathways (see FIGURE 2) (Carpenter, G., *FASEB J.* 6:3283-3289, 1992; Sierke, S. and Koland, J. *Biochem.* 32:10102-10108, 1993). The SH2 domains are responsible for coupling the activated growth-factor receptors to cellular responses which include alterations in gene expression, and ultimately cellular proliferation. Thus, inhibitors that will selectively block the effects of activation of specific RTKs (excluding IGFR and FGFR) expressed on the surface of synoviocytes are predicted to be effective in blocking cartilage degradation after arthroscopy procedures.

At least 20 cytosolic proteins have been identified that contain SH2 domains and function in intracellular signaling. The distribution of SH2 domains is not restricted to a particular protein family, but found in several classes of proteins, protein kinases, lipid kinases, protein phosphatases, phospholipases, Ras-controlling proteins and some transcription factors. Many of the SH2-containing proteins have known enzymatic activities while others (Grb2 and Crk) function as "linkers" and "adapters" between cell surface receptors and "downstream" effector molecules (Marengere, L., et al., *Nature* 369:502-505, 1994). Examples of proteins containing SH2 domains with enzymatic activities that are activated in signal transduction

include, but are not limited to, the src subfamily of protein tyrosine kinases (src (pp60^{c-src}), abl, lck, fyn, fgr and others), phospholipaseC γ (PLC γ), phosphatidylinositol 3-kinase (PI-3-kinase), p21-ras GTPase activating protein (GAP) and SH2 containing protein tyrosine phosphatases (SH-PTPases) (Songyang, et al., Cell 72, 767-778, 1993). Due to the central role these various SH2-proteins occupy in transmitting signals from activated cell surface receptors into a cascade of additional molecular interactions that ultimately define cellular responses, inhibitors which block specific SH2 protein binding, e.g., c-src) are desirable as agents with potential therapeutic applications in cartilage protection.

In addition, the regulation of many immune/inflammatory responses is mediated through receptors that transmit signals through non-receptor tyrosine kinases containing SH2 domains. T-cell activation via the antigen specific T-cell receptor (TCR) initiates a signal transduction cascade leading to lymphokine secretion and T-cell proliferation. One of the earliest biochemical responses following TCR activation is an increase in tyrosine kinase activity. In particular, neutrophil activation is in part controlled through responses of the cell surface immunoglobulin G receptors. Activation of these receptors mediates activation of unidentified tyrosine kinases which are known to possess SH2 domains. Additional evidence indicates that several src-family kinases (lck, blk, fyn) participate in signal transduction pathways leading from cytokine and integrin receptors and hence may serve to integrate stimuli received from several independent receptor structures. Thus, inhibitors of specific SH2 domains have the potential to block many neutrophil functions and serve as anti-inflammatory mediators.

Efforts to develop drugs targeted to SH2 domains currently are being conducted at the biochemical *in vitro* and cellular level. Should such efforts be successful, it is theorized that the resulting drugs would be useful in the practice of the present invention.

III. Synergistic Interactions Derived From Therapeutic Combinations Of Anti-pain and/or Anti-inflammation Agents And Other Agents Used In Chondroprotective Solutions

Given the complexity of the disease process associated with inflammation and loss of cartilage homeostasis after arthroscopic therapeutic procedures and the multiplicity of molecular targets involved, blockade or inhibition of a single molecular target is unlikely to provide adequate efficacy in preventing cartilage degradation and the development of osteoarthritis. Indeed, a number of animal

studies targeting different individual molecular receptors and or enzymes have not proven effective in animal models or have not yielded efficacy in clinical trials to date. Therefore, a therapeutic combination of drugs acting on distinct molecular targets and delivered locally appears desirable for clinical effectiveness in the therapeutic approach to cartilage protection. As described below, the rationale for this synergistic molecular targeted therapy is derived from recent advances in understanding fundamental biochemical mechanisms by which synoviocyte and chondrocyte cells in the synovium and cartilage transmit and integrate stimuli to which they are exposed during arthroscopic procedures.

10 "Crosstalk" and Convergence in Major Signaling Pathways

The molecular switches responsible for cell signaling have been traditionally divided into major discrete signaling pathways, each comprising a distinct set of protein families that act as transducers for a particular set of extracellular stimuli and mediating distinct cell responses. One such pathway transduces signals from neurotransmitters and hormones through G-protein coupled receptors (GPCRs) to produce contractile responses which include the production of inflammatory mediators, such as PGE₂. The GPCRs couple to intracellular targets through activation of trimeric G proteins (see FIGURE 2). Examples of signaling molecules involved in activation of synoviocytes and chondrocytes through the GPCR pathway are histamine, bradykinin, serotonin and ATP. A second major pathway transduces signals from pro-inflammatory cytokines, such as IL-1, through a kinase cascade and NF- κ B protein into regulation of gene expression and the production of catabolic cytokines and other catabolic factors, including NO.

Signals transmitted from neurotransmitters and hormones stimulate either of two classes of receptors: GPCRs, composed of seven-helix transmembrane regions, or ligand-gated ion channels. "Downstream" signals from both kinds of receptors converge on controlling the concentration of cytoplasmic Ca²⁺ (see FIGURE 3). Each GPCR transmembrane receptor activates a specific class of trimeric G proteins, including G_q, G_i or many others. G_q subunits activate phospholipase C γ , resulting in activation of protein kinase C (PKC) and an increase in the levels of cytoplasmic calcium (FIGURE 3). In turn, elevated intracellular calcium leads to the activation of cPLA₂ and the production of arachidonic acid (AA). The AA serves as a substrate for COX in both synoviocytes and chondrocytes, leading to the production of PGE₂. PKC activation also results in activation of MAP kinase leading to activation of NF- κ B and, in cells and tissues which have been primed by exposure to pro-inflammatory

cytokines, modulates increased gene expression of proteins involved in cartilage catabolism.

Pro-inflammatory cytokine signaling, such as mediated by both IL-1 and TNF- α through their distinct cognate receptors, also converges on regulation of cell gene expression. The signal transduction pathways utilized by these distinct receptors, employ distinct kinases that are proximal to the receptors but the signaling pathways subsequently converge at the level of MAP kinases (FIGURE 3 and 4). Signal transduction depends upon phosphorylation of residues in a cascade of kinases, including "downstream" enzymes such as p38 MAP kinase. Activation of the IL-1-receptor and TNF-receptor also leads to stimulation of MAP kinase, common steps shared by the Gq coupled GPCRs (see FIGURE 3). It is now recognized that ligand-independent "crosstalk" can transactivate kinase pathways in response to costimulation of specific GPCRs and cytokines such as IL-1, leading to synergistic cellular responses (see FIGURE 3). Thus, a combination of selective inhibitors which blocks transactivation of a common signaling pathway (as shown in FIGURES 1 and 2) leading to increased gene expression of pro-inflammatory cytokines, iNOS, COX-2, and MMPs will act synergistically to prevent inflammation and cartilage degradation after arthroscopic surgical procedures.

IV SUMMARY

From the molecular and cellular mechanisms of action defined for these chondroprotective agents, these compounds are expected to exhibit chondroprotective action when applied perioperatively in an irrigation solution (in combination with other chondroprotective agents or in combination with other anti-pain and anti-inflammation agents described herein) or otherwise administered directly to the joint via infusion or injection. In particular, these agents are expected to be effective drugs when delivered by an irrigation solution during an arthroscopic surgical procedure. Each metabolically active chondroprotective agent may be delivered in combination with one or more other chondroprotective agents, including small molecule drugs, peptides, proteins, recombinant chimeric proteins, antibodies, oligonucleotides or gene therapy vectors (viral and nonviral), to the spaces of the joint. For example, a drug such as a MAPK inhibitor can exert its actions on any cells associated with the fluid spaces of the joint and structures comprising the joint that are involved in the normal function of the joint or are present due to a pathological condition. These cells and structures include, but are not limited to: synovial cells, including both Type A fibroblast and type B macrophage cells; the cartilaginous components of the joint

such as chondroblasts and chondrocytes; cells associated with bone, including periosteal cells, osteocytes, osteoblasts, osteoclasts; inflammatory cells including lymphocytes, macrophages, mast cells, monocytes, eosinophils; and other cells including endothelial cells, smooth muscle cells, fibroblasts and neural cells; and combinations of the above.

This aspect of the present invention also provides for formulations of the active therapeutic agent(s) which may be delivered in a formulation useful for introduction and administration of the drug into the joint that would enhance the delivery, uptake, stability or pharmacokinetics of the chondroprotective agent(s). Such a formulation may include, but is not limited to, microparticles, microspheres or nanoparticles composed of proteins, liposomes, carbohydrates, synthetic organic compounds, or inorganic compounds. The present invention provides for the delivery of a combination of chondroprotective agents, or one or more chondroprotective agents with one or more anti-pain and/or anti-inflammation agents present either as multiple pharmaceutically active substances within a homogeneous vehicle (e.g., a single encapsulated microsphere) or as a discrete mixture of individual delivery vehicles (e.g., a group of microspheres encapsulating one or more agents). Examples of formulation molecules include, but are not limited to, hydrophilic polymers, polycations (e.g. protamine, spermidine, polylysine), peptide or synthetic ligands and antibodies capable of targeting materials to specific cell types, gels, slow release matrices, soluble and insoluble particles, as well as formulation elements not listed.

In one aspect, the present invention provides for the local delivery of a combination of two or more chondroprotective agents, or one or more chondroprotective agents in combination with one or more anti-pain and/or anti-inflammation agents, alone or in combination with one or more anti-pain and/or anti-inflammatory agents, via an irrigation solution, an infusion containing the drugs which are present at therapeutically effective low concentrations and which enables the drugs to be delivered directly to the affected tissue or joint. The drug-containing infusion or irrigation solution may be employed pre-operatively and/or intra-operatively and/or post-operatively in connection with a surgical procedure or may be administered at other times not related to surgical procedures. Other conventional methods used for drug delivery have required systemic (e.g., intramuscular, intravenous, subcutaneous) administration which necessitate higher concentrations of drugs (and higher total dose) to be administered to the patient in order to achieve significant therapeutic concentrations in the targeted joint. Systemic administration

also results in high concentrations in tissues other than the targeted joint which is undesirable and, depending on the dose, may result in adverse side effects. These systemic methods subject the drug to second-pass metabolism and rapid degradation, thereby limiting the duration of the effective therapeutic concentration. Since the combination of chondroprotective agents (with or without one or more anti-pain and/or anti-inflammatory agents) are administered directly to the joint by infusion or by irrigation, vascular perfusion is not required to carry the drug to the targeted tissue. This significant advantage allows for the local delivery of a lower therapeutically effective total dose for a variety of chondroprotective drugs.

V. Method of Application

The solutions of the present invention has applications for a variety of operative/interventional procedures, including surgical, diagnostic and therapeutic techniques. The combination of chondroprotective agents of the invention may be administered by injection or by irrigation. For solutions for injection, the amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the patient to be treated, the nature of the active agents in the solution and the particular mode of administration. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex and diet of the patient, time of administration, route of administration, rate of excretion of the drug combination, and the severity of the particular disease undergoing therapy.

Injectable preparations, for example, sterile injectable aqueous or oleagenous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1/3-propanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The solutions for injection of the invention may be administered in connection with an arthroscopic surgical procedure or at any time determined to be desirable by a physician directing patient care.

The irrigation solutions of the invention may be perioperatively applied during arthroscopic surgery of anatomic joints. As used herein, the term "perioperative" encompasses application intraprocedurally, pre- and intraprocedurally, intra- and postprocedurally, and pre-, intra- and postprocedurally.

5 Preferably the solution is applied preprocedurally and/or postprocedurally as well as intraprocedurally. Such procedures conventionally utilize physiologic irrigation fluids, such as normal saline or lactated Ringer's, applied to the surgical site by techniques well known to those of ordinary skill in the art. The method of the present invention involves substituting the anti-pain/anti-inflammatory/chondroprotective
10 irrigation solutions of the present invention for conventionally applied irrigation fluids. The irrigation solution is applied to the wound or surgical site prior to the initiation of the procedure, preferably before tissue trauma, and continuously throughout the duration of the procedure, to preemptively block pain and inflammation, and cartilage degradation. As used herein throughout, the term
15 "irrigation" is intended to mean the flushing of a wound or anatomic structure with a stream of liquid. The term "application" is intended to encompass irrigation and other methods of locally introducing the solution of the present invention, such as introducing a gellable version of the solution to the operative site, with the gelled solution then remaining at the site throughout the procedure. As used herein
20 throughout, the term "continuously" is intended to also include situations in which there is repeated and frequent irrigation of wounds at a frequency sufficient to maintain a predetermined therapeutic local concentration of the applied agents, and applications in which there may be intermittent cessation of irrigation fluid flow necessitated by operating technique.

25 The concentrations listed for each of the agents within the solutions of the present invention are the concentrations of the agents delivered locally, in the absence of metabolic transformation, to the operative site in order to achieve a predetermined level of effect at the operative site. It is understood that the drug concentrations in a given solution may need to be adjusted to account for local dilution upon delivery.
30 Solution concentrations are not adjusted to account for metabolic transformations or dilution by total body distribution because these circumstances are avoided by local delivery, as opposed to oral, intravenous, subcutaneous or intramuscular application.

Arthroscopic techniques for which the present solution may be employed include, by way of non-limiting example, partial meniscectomies and ligament
35 reconstructions in the knee, shoulder acromioplasties, rotator cuff debridements, elbow synovectomies, and wrist and ankle arthroscopies. The irrigation solution is

continuously supplied intraoperatively to the joint at a flow rate sufficient to distend the joint capsule, to remove operative debris, and to enable unobstructed intra-articular visualization.

5 Suitable arthroscopic irrigation solutions for inhibition of cartilage degradation and control of pain and inflammation during such arthroscopic techniques are provided in Examples 1-4 herein below.

10 In each of the solutions of the present invention, the agents are included in low concentrations and are delivered locally in low doses relative to concentrations and doses required with conventional methods of drug administration to achieve the desired therapeutic effect. It is impossible to obtain an equivalent therapeutic effect by delivering similarly dosed agents via other (i.e., intravenous, subcutaneous, intramuscular or oral) routes of drug administration since drugs given systemically are subject to first- and second-pass metabolism and are often rapidly cleared from the system circulation.

15 Practice of the present invention should be distinguished from conventional intra-articular injections of opiates and/or local anesthetics at the completion of arthroscopic or "open" joint (e.g., knee, shoulder, etc.) procedures. The solution of the present invention is used for continuous infusion throughout the surgical procedure to provide preemptive inhibition of pain and inflammation. In contrast, the high concentrations necessary to achieve therapeutic efficacy with a constant infusion of currently used local anesthetics can result in profound systemic toxicity.

20 Upon completion of the procedure of the present invention, it may be desirable to inject or otherwise apply a higher concentration of the same chondroprotective agent(s) and/or pain and/or inflammation inhibitors as used in the irrigation solution at the operative site, as an alternative or supplement to opiates. In addition, the direct injection of combinations of chondroprotective agents may be desirable, as described in detail herein. A suitable chondroprotective solution for injection is provided in Example 5 herein below.

EXAMPLES

30 The following are several formulations in accordance with the present invention suitable for certain operative procedures followed by a summary of three clinical studies utilizing the agents of the present invention.

Example 1
Irrigation Solution for Arthroscopy

5 The following composition is suitable for use in anatomic joint irrigation during arthroscopic procedures. Each drug is solubilized in a carrier fluid containing physiologic electrolytes, such as normal saline or lactated Ringer's solution, as are the remaining solutions described in subsequent examples.

<u>Class of Agent</u>	<u>Drug</u>	<u>Concentration (Nanomolar)</u>
MAP Kinase Inhibitor	SB203580	200
Matrix Metalloproteinase Inhibitor	U-24522	200
TGF- β Agonist	TGF- β 2	200

Example 2
Alternative Irrigation Solution for Arthroscopy

10 The following composition is an alternate formulation suitable for use in anatomic joint irrigation during arthroscopic procedures.

<u>Class of Agent</u>	<u>Drug</u>	<u>Concentration (Nanomolar)</u>
MAP Kinase Inhibitor	SB203580	200
Nitric Oxide Synthase Inhibitor	L-NIL	1,000
Interleukin Receptor Agonist	IL-10	100

Example 3
Alternate Irrigation Solution

15 The following drugs and concentration ranges in solution in a physiologic carrier fluid are suitable for use in the present invention.

<u>Class of Agent</u>	<u>Drug</u>	<u>Concentration (Nanomolar)</u>
MAP Kinase Inhibitor	SB242235	200
Nitric Oxide Synthase Inhibitor	L-NIL	10,000
TGF- β Agonist	TGF- β 2	100

Example 4
Alternate Irrigation Solution

The following composition is also useful in the present invention.

<u>Class of Agent</u>	<u>Drug</u>	<u>Concentration (Nanomolar)</u>
MAP Kinase Inhibitor	SB242235	200
MMP Inhibitor	U-24522	200

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Example 5
Chondroprotective Solution for Injection

The following composition is suitable for injection into an anatomic joint. Each drug is solubilized in a carrier fluid containing physiologic electrolytes, such as normal saline or lactated Ringer's solution. A dosage of 20 ml of the solution is suitable for administration to a patient.

20

<u>Class of Agent</u>	<u>Drug</u>	<u>Concentration</u>
BMP Receptor Agonist	BMP-7	100 ng/ml
Nitric Oxide Synthase Inhibitor	1,3 PBIT	4.4 µg/ml
TGF-β Agonist	pyrrolidine- dithiocarbamate	16.4 µg/ml

Example 6

Synergistic stimulation of a rapid PGE2 burst upon exposure to IL-1 and GPCR agonists.

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Fibroblast-like synoviocytes exhibit characteristics of inflammatory cells and seem to be crucial regulators of joint inflammation and cartilage degradation. A synoviocyte cell culture model system was used to characterize the synergistic interactions between IL-1 and non-cytokine inflammatory mediators which are important in modulating the destruction of joint tissue, including damage that occurs

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as a consequence of tissue injury during arthroscopic surgery. Experiments were conducted to investigate G-protein coupled receptor (GPCR) agonists (histamine, bradykinin and isoproterenol) on the regulation of cytokine and prostanoid production in cultured human synovial fibroblasts and to characterize the activities of ketoprofen in this system. The kinetics of induction of prostaglandin E2 (PGE₂), interleukin-6 (IL-6) and interleukin-8 (IL-8) in response to stimulation with interleukin-1 (IL-1) are described. The ability of GPCR ligands to potentiate cytokine production following IL-1 priming was investigated.

In Examples 6-8, the following experimental methods and materials were employed unless otherwise indicated.

Cell Culture. Synovial tissue was obtained from osteoarthritis patients undergoing joint replacement surgery through the Clinical Research Center, MacNeal Hospital, and transported to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml). The synovium was dissected and minced with scissors, and plated as explants in culture medium composed of DMEM containing L-glutamine (2 mM), heat inactivated fetal bovine serum (10% v/v), plus antibiotics. Cultures were housed at 37° C in a humidified atmosphere of 5% CO₂. Adherent synovial cells grew out of the explants within 2-3 weeks, and were passaged by trypsinization. Seed cultures were fed twice weekly and were passaged at confluency. Experiments were performed on cells from passages 3-8. Experimental cultures were plated into 35 mm dishes at a density of 7.5×10^3 cells/cm² in 2 ml culture medium. Cultures were grown to near confluency for experiments, and contained $2.3 \pm 0.3 \times 10^5$ cells (mean \pm S.E.M., n=3), and 104 ± 13 µg protein (n=10). The growth medium was replaced twice weekly.

Experimental Treatments. One day prior to initiation of experimental treatments, medium was changed to experimental growth medium composed of DMEM containing 2% heat-inactivated fetal bovine serum, plus L-glutamine and antibiotics as above, to render the cells quiescent. The next day, cultures were primed by addition of specified concentrations of IL-1 or additional ligands to the conditioned growth medium for 12-24 hr intervals, as indicated. In some experiments, conditioned growth medium was collected for analysis following priming with IL-1. Acute experimental treatments were performed after this priming interval, as follows. Cultures were removed from the incubator, washed three times with 2 ml aliquots of Locke's physiological buffer (LB composition in mM: NaCl, 154; KCl, 2.6; KH₂PO₄, 2.15; K₂HPO₄, 0.85; MgCl₂, 5; CaCl₂, 2; D-glucose, 10;

HEPES, 10; pH 7.4, BSA, 0.1% w/v), and then equilibrated with an additional aliquot of LB containing specified ligands for 10 min on a 37° bath. This solution was removed by aspiration and replaced with a fresh buffer aliquot containing indicated ligands for specified time intervals at 37°. Pharmacological inhibitors typically were added during the 10 min preincubation interval, and agonists plus the specified inhibitors were present during the 3 min challenge interval.

Measurement of prostaglandin E2. Following indicated treatment protocols, aliquots of culture supernatant (1 ml) were collected and rapidly frozen in liquid nitrogen. Samples were stored at -80° until processing. Aliquots of culture supernatant were analyzed by competitive binding radioimmunoassay as specified by the manufacturer (Sigma Chemical Co.), using an antibody with equivalent reactivity toward prostaglandins E2 and E1. For quantitation, a standard curve was prepared with each assay using fixed concentrations of [³H]prostaglandin E2, and increasing concentrations of authentic competing prostaglandin E2.

Measurement of IL-6. Production of the cytokine, IL-6, was also measured in aliquots of supernatant culture media which had been stored frozen at -80 ° C. IL-6 was measured by sandwich ELISA with alkaline phosphatase detection as described by the manufacturer (Pharmingen) and quantitated using standard curves prepared with the respective pure recombinant human cytokines. Experimental determinations were performed on duplicate cultures.

Assays for [³H]thymidine Incorporation and MTT

Synoviocyte cell lines were routinely evaluated for competence to proliferate in response to IL-1, measured as incorporation of [³H]thymidine (Kimball & Fisher, 1988). In this preparation, maximally effective concentrations of IL-1 stimulate [³H]thymidine incorporation by 10-20 fold compared to quiescent cultures maintained in 2% serum (data not shown).

Data analysis. Immunoassays were routinely performed in duplicate aliquots from each culture. Experimental determinations were performed on duplicate or triplicate cultures. Each experiment was repeated in at least two cell lines. Nonlinear regression curve fitting and statistical analyses were performed using Graph-PAD Prism software (San Diego, CA).

Materials. Cell culture: Cell culture media were obtained from Sigma or Gibco/BRL. Fetal bovine serum was from Atlanta Biologicals Inc. (Norcross, GA). Drugs: Recombinant human interleukin-1 was obtained from Genzyme (Cambridge, MA). Ketoprofen was provided by Omeros Medical Systems, Inc. (Seattle, WA).

Amitriptyline, forskolin, 5-hydroxytryptamine, isoproterenol, Bradykinin, histamine, and prostaglandin E2 were from Sigma. Radiochemicals: [³H]Prostaglandin E2, was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other reagents were obtained in the highest purity available from standard commercial suppliers.

The effect of GPCR agonists, histamine and bradykinin, on PGE2 production in human synovial cells was measured with and without prior stimulation to IL-1 to assess the functional interactions between agonists mediating a common pharmacological effect through these different classes of receptors. Overnight exposure of cultured human synovial fibroblasts to IL-1 (10 U/ml) results in a delayed (4 hrs) and sustained large enhancement of PGE2 production, which can be measured by radioimmunoassay as increased PGE2 in the culture supernatant. The progressive increase in PGE2 production during prolonged IL-1 treatment (16-24 hr) has been shown to arise from the coordinated upregulated expression of cPLA2 and the COX-2 (Crofford, 1984, Hulkower et al., 1984). Cultures which have been primed by overnight exposure to IL-1 respond to subsequent challenges with maximally effective concentrations of histamine (100 μ M) or bradykinin (1 μ M) with additional rapid (minutes) and robust production of PGE2. Representative data for the time course of PGE2 production in response to histamine or bradykinin stimulation are shown in Figure 7. Under these conditions, histamine elicits a 5-10 fold increase in PGE2 production compared to IL-1 primed cells receiving no GPCR agonist addition. Bradykinin elicits a 10-15 fold increase. The absolute quantity of PGE2 produced during the brief 2 min agonist challenge approaches or exceed quantities that are cumulatively produced during the entire 18 hr IL-1 priming interval. This is remarkable insofar as Fig. 7 shows that the vast majority of the histamine-elicited burst in PGE2 production occurs within the initial 2 min period since minimal additional accumulation is observed over the subsequent 60 min period. The bradykinin-stimulated PGE2 response continues to increase (2-fold) over the same time period. In the absence of IL-1 priming, naive synoviocytes show no detectable PGE2 production in response to stimulation with either GPCR agonist alone. Under conditions of IL-1 priming, histamine and bradykinin both synergistically potentiated PGE2 release.

Using cultured synovial fibroblasts from osteoarthritis patients, we found time-dependent synergistic interactions between the pro-inflammatory cytokine, IL-1, and physiologically relevant G-protein coupled receptors on PGE2 production, and evaluated the actions of target therapeutic agents. GPCR agonists acting through

endogenous synoviocyte receptors which are coupled to increases in intracellular calcium, inositol phosphates and PKC signaling pathways rapidly and dramatically amplify PGE2 production in cells previously primed by IL-1. COX inhibitors effectively attenuated both the agonist-elicited rapid burst and the long-term accumulation of PGE2. Thus, different GPCR and IL-1 pathways for intracellular signal transduction synergistically interact to bring about either rapid or slower, long-term regulation of PGE2 responses.

The synergism between IL-1 and calcium-regulatory GPCRs in synoviocytes that produce the rapid PGE2 burst may in part be explained by the rapid augmentation of arachidonic acid release, a measure of cPLA2 activation in many cell types. In addition to inducing COX-2 expression, IL-1 increases expression of cPLA2 (Hulkower et al., 1994). These two proteins act together to provide free arachidonic acid substrate for COX-2. The upregulation of the key eicosonoid metabolizing enzymes induced by IL-1, combined with the ability of the GPCR ligands to activate arachidonate release, would therefore be predicted to increase overall substrate flux through prostanoid synthesis. cPLA2 is the only known PLA2 that exhibits functional properties indicative of receptor regulation and is likely to be involved in eicosonoid production and intracellular signaling. Since cPLA2 is activated by increasing calcium concentrations for full activity and bradykinin B2 and histamine H1 receptor activation is coupled to mobilization of intracellular calcium, this is likely the predominant factor regulating the rapid agonist-stimulated burst in PGE2 production. Finally, the very rapid and transient increase in cytoplasmic calcium triggered by B2 or H1 receptor activation is similar to the kinetics known for cPLA2 activation, arachidonic acid release, and the observed PGE2 burst.

Example 7

Inhibition of PGE2 burst formation by cyclooxygenase inhibitors.

The actions of ketoprofen, a cyclooxygenase inhibitor, to attenuate PGE2 formation were determined by co-incubation with IL-1 during prolonged exposure (16 hr); and by brief pre-incubation prior to a subsequent GPCR agonist challenge interval, as shown in Figure 8. Addition of specified concentrations of ketoprofen during overnight priming with IL-1 abolishes PGE2 formation, with $IC_{50} = 4.5 \pm 0.8$ nM determined by nonlinear regression analysis (mean \pm SEM, n=4 synoviocyte cell lines). Similar determinations (data not shown) were performed with the cyclooxygenase inhibitors etodolac ($IC_{50} = 15.2 \pm 4.6$ nM, n=4), ketorolac (2.2 ± 0.4 nM, n=4), and indomethacin (3.2 ± 1.5 nM, n=2).

Figure 8 also shows the ketoprofen concentration-dependent inhibition of the agonist-elicited PGE₂ burst in response to a challenge by 100 μ M histamine (IC_{50} = 3.4 ± 0.2 nM, n=3) or 1 μ M bradykinin (IC_{50} = 9.5 ± 2.0 nM, n=3) in synoviocytes primed overnight with IL-1 (10 U/ml). These values are comparable to those observed for ketoprofen inhibition during overnight IL-1 induction of PGE₂. This result demonstrates that the onset of inhibition by the COX inhibitor occurs within the 10 min pretreatment interval prior to GPCR agonist addition, consistent with a direct, reversible inhibition of the COX activity and not due a mechanism linked to changes in the expression levels of the prostanoid regulatory enzymes. This immediate inhibitory effect also provides a basis for the immediate effectiveness of this drug when delivered locally to the intra-articular in an irrigation solution during arthroscopic surgery.

Example 8

Induction of IL-6 production by IL-1 and GPCR agonists and inhibition by ketoprofen.

The kinetics of induction of interleukin-6 in response to stimulation with IL-1 are described. Synoviocyte cultures were exposed to the indicated treatments with IL-1 plus either histamine to activate signaling through inositol trisphosphate (InsP₃)/protein kinase C pathway or isoproterenol to activate increases in intracellular cAMP. Production of PGE₂, IL-6, and IL-8 were measured in the culture supernatants following 1, 2, 4, 6, and 24 hr treatments. In this experiment, each treatment interval was performed in a separate culture. In the above treatment regime, production of IL-6 was robustly increased by IL-1 following 24-hr exposure, but no IL-6 was detected within the initial 6 hr interval. IL-6 production in response to IL-1 was not augmented further by addition of histamine, and histamine alone failed to stimulate IL-6 production. IL-1 also produced a significant elevation of IL-8 (2000 pg/ml), which was first measurable at 6 hr of treatment. IL-8 production was sustained and greatly increased at 24-hr exposure to IL-1.

The effect of ketoprofen on the induction of cytokine production by IL-1 and GPCR agonists was examined. The protocol also tested the effects of IL-1 concentration dependence on the IL-6 steady state induction. Synoviocyte cultures were exposed to indicated concentrations of IL-1 and GPCR agonists. Culture supernatants were collected and replaced with fresh media aliquots containing the same agonist additions at 8-hr intervals. PGE₂, IL-6, and IL-8 in the supernatants were assayed as described.

Data for IL-6 production are shown in Fig. 9 which shows IL-6 production at 16 hr (corresponding to treatment interval from 8-16 hr) in the presence of indicated concentrations of IL-1 plus added ligand. Addition of histamine or isoproterenol does not enhance IL-6 production compared to IL-1 alone. At 1.0 pg/ml IL-1, ketoprofen causes a partial ($\leq 50\%$) inhibition of IL-1-elicited IL-6 production. Furthermore, ketoprofen inhibited IL-6 production in the histamine or isoproterenol/IL-1 co-stimulated samples.

The synoviocyte cell culture model system was used to characterize the synergistic interactions between IL-1 and non-cytokine inflammatory mediators which are important in modulating the destruction of joint tissue, including damage that occurs as a consequence of tissue injury during arthroscopic surgery. The results can be summarized as follows: (1) IL-1 induces large increases in PGE₂, IL-6, and IL-8 in cultured synoviocytes, whereas quiescent cultures do not produce detectable quantities of these mediators, (2) the induction of PGE₂ occurs most rapidly and results in release of PGE₂ to the culture supernatant at 4 hr, followed by IL-8 at 6 hr, and IL-6 at longer intervals, and (3) all three mediators remain elevated in the culture supernatant following 24 hr IL-1 exposure.

In contrast to their actions on PGE₂ production, the GPCR agonists do not enhance IL-1 induction of IL-6 or IL-8 and also do not increase IL-6 and IL-8 release following priming with IL-1. IL-1 induction of IL-6 and IL-8 appears to be reinforced by the concomitant induction of PGE₂ since ketoprofen reduces the production of these cytokines in response to IL-1. This result indicates that ketoprofen could provide a therapeutic chondroprotective effect when delivered to the joint during surgical procedures.

Taken together, these results demonstrate interactions between specific G-coupled receptor signaling pathways and the activation of synoviocytes by pro-inflammatory stimulation with IL-1. A similar mechanism is expected to be operative in chondrocytes. These interactions provide a means of integrating and modulating pro-inflammatory responses of synoviocytes and chondrocytes depending on inputs from other autocrine or neurotransmitter receptor systems within the joint. These findings underscore the rationale and potential clinical benefit of therapeutic interventions which target inhibition of G-protein coupled receptors that mediate signalling through calcium mobilization, phosphoinositide hydrolysis and PKC activation and are coupled to increases in production of PGE₂ in arthroscopic surgery. These receptors on synoviocytes and chondrocytes include histamine H₁, bradykinin, Substance P, 5HT₂, and the purinergic P₂Y receptors.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of inhibiting cartilage degradation in a joint of a patient, comprising:

delivering to the joint a composition in solution comprising a therapeutically effective amount of a first chondroprotective agent and a therapeutically effective amount of at least one second agent selected from the group consisting of second chondroprotective agents, inhibitors of pain, inhibitors of inflammation, and mixtures thereof.

2. The method of Claim 1, wherein the solution is delivered to the joint perioperatively during a surgical procedure.

3. The method of Claim 2, comprising irrigating the joint with the solution during the surgical procedure.

4. The method of Claim 2, wherein the procedure is an arthroscopic surgical procedure and the solution is delivered to the joint prior to, during or after the surgical procedure.

5. The method of Claim 2, wherein the procedure is an arthroscopic surgical procedure and the solution is delivered to the joint prior to, during and after the surgical procedure.

6. The method of Claim 4, wherein a sufficient amount of the solution is delivered to the joint after the surgical procedure so that a bolus of the solution remains in the synovial capsule of the patient following the surgical procedure.

7. The method of Claim 1, wherein the first chondroprotective agent is an anabolic chondroprotective agent and the second agent is a second chondroprotective agent that is an inhibitor of cartilage catabolism, and the solution is delivered to the joint by injection.

8. The method of Claim 6, wherein the first chondroprotective agent is an anabolic cytokine selected from the group consisting of interleukin (IL) agonists, members of the transforming growth factor- β superfamily, insulin-like growth factors and fibroblast growth factors.

9. The method of Claim 7, wherein the first chondroprotective agent is selected from the group consisting of IL-4, IL-10, IL-13, TGF β 1, TGF β 2, BMP-2, BMP-4, BMP-7, IGF-1 and bFGF).

10. The method of Claim 7, wherein the second chondroprotective agent is an inhibitor of the activity or the expression of a pro-inflammatory molecular target selected, the inhibitor being selected from the group consisting of IL-1 receptor antagonists, TNF- α receptor antagonists, cyclooxygenase-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase inhibitors, and nuclear factor kB inhibitors.

11. The method of Claim 1, wherein the solution comprises one or more pain or inflammation inhibitory agents, the pain or inflammation inhibitory agents being selected to act on a plurality of differing molecular targets, wherein the solution is applied locally and perioperatively to the surgical site.

12. The method of Claim 11, wherein the pain or inflammation inhibitory agents are selected from the group consisting of serotonin receptor antagonists, serotonin receptor agonists, histamine receptor antagonists, bradykinin receptor antagonists, kallikrein inhibitors, tachykinin receptor antagonists, calcitonin gene-related peptide (CGRP) receptor antagonists, interleukin receptor antagonists, inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, prostanoid receptor antagonists, leukotriene receptor antagonists, opioid receptor agonists, purinoceptor agonists and antagonists, adenosine triphosphate (ATP)-sensitive potassium channel openers, and calcium channel antagonists.

13. The method of Claim 1, wherein the solution is locally applied prophylactically to the joint of a patient or to the joint of a patient suffering from articular cartilage degradation.

14. The method of Claim 2, wherein the perioperative delivery of the solution comprises intraprocedural delivery together with preprocedural or postprocedural delivery of the solution.

15. The method of Claim 2, wherein the perioperative application of the solution comprises preprocedural, intraprocedural and postprocedural application of the solution.

16. A method of inhibiting cartilage degradation in a joint of a patient, comprising:

delivering to the joint a composition in solution comprising a therapeutically effective amount of at least one chondroprotective agent and at least one inhibitor of pain or inflammation in a liquid carrier.

17. The method of Claim 16, wherein the solution is delivered to the joint perioperatively during a surgical procedure.

18. The method of Claim 17, comprising continuously irrigating the joint with the solution during the surgical procedure.

19. The method of Claim 17, wherein the procedure is an arthroscopic surgical procedure and the solution is delivered to the joint prior to, during or after the surgical procedure.

20. The method of Claim 17, wherein the procedure is an arthroscopic surgical procedure and the solution is delivered to the joint prior to, during and after the surgical procedure.

21. The method of Claim 17, wherein a sufficient amount of the solution is delivered to the joint after the surgical procedure so that a bolus of the solution remains in the synovial capsule of the patient following the surgical procedure.

22. The method of Claim 16, wherein the chondroprotective agent is selected from the group consisting of interleukin 1 receptor antagonists, tumor necrosis factor receptor antagonists, interleukin receptor agonists, TGF- β superfamily receptor agonists, COX-2 inhibitors, MAP kinase inhibitors, inhibitors of matrix metalloproteinases, inhibitors of NF- κ B, nitric oxide synthase inhibitors, agonists and antagonists of integrin receptors, inhibitors of the protein kinase C family, inhibitors of the protein tyrosine kinase family, modulators of protein tyrosine phosphatases and inhibitors of protein src homology 2 domains.

23. The method of Claim 16, wherein the inhibitor of pain or inflammation is selected from the group consisting of serotonin receptor antagonists, serotonin receptor agonists, histamine receptor antagonists, bradykinin receptor antagonists, kallikrein inhibitors, tachykinin receptor antagonists, calcitonin gene-related peptide (CGRP) receptor antagonists, interleukin receptor antagonists,

inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, prostanoid receptor antagonists, leukotriene receptor antagonists, opioid receptor agonists, purinoceptor antagonists, and calcium channel antagonists.

24. The method of Claim 16, wherein the solution is locally applied prophylactically to the joint of a patient or to the joint of a patient suffering from articular cartilage degradation.

25. The method of Claim 17, wherein the perioperative delivery of the solution comprises intraprocedural delivery together with preprocedural or postprocedural delivery of the solution.

26. The method of Claim 17, wherein the perioperative application of the solution comprises preprocedural, intraprocedural and postprocedural application of the solution.

27. A method of inhibiting cartilage degradation, comprising delivering to a potential cartilage degradation site a solution comprising at least two chondroprotective agents in a liquid carrier, wherein at least one of said agents is an anabolic chondroprotective agent and at least another one of said agents is an inhibitor of cartilage catabolism.

28. A solution for use in the inhibition of cartilage degradation, comprising at least one anabolic chondroprotective agent and a second chondroprotective agent that is an inhibitor of cartilage catabolism.

29. The solution of Claim 28, wherein the anabolic chondroprotective agent is an anabolic cytokine selected from the group consisting of interleukin (IL) agonists, members of the transforming growth factor- β superfamily, insulin-like growth factors and fibroblast growth factors.

30. The solution of Claim 29, wherein the anabolic chondroprotective agent is selected from the group consisting of IL-4, IL-10, IL-13, TGF β , BMP-7, IGF-1 and bFGF).

31. The solution of Claim 28, wherein the second chondroprotective agent is an inhibitor of the activity or the expression of a pro-inflammatory molecular target selected, the inhibitor being selected from the group consisting of IL-1 receptor

antagonists, TNF- α receptor antagonists, cyclooxygenase-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase inhibitors, and nuclear factor kappaB inhibitors.

32. A solution for use in the inhibition of cartilage degradation and preemptive inhibition of pain and inflammation at a joint of a patient during a surgical procedure, comprising at least one chondroprotective agent and at least one pain and inflammation inhibitory agent in a liquid carrier, the concentration of each agent within the solution being the concentration of that agent which is desired to be delivered locally, in the absence of metabolic transformation, to a joint in order to achieve a predetermined level of inhibition of cartilage degradation, pain and inflammation at the surgical site.

33. The solution of Claim 32, wherein the chondroprotective agent is selected from the group consisting of interleukin-1 receptor antagonists, tumor necrosis factor receptor antagonists, interleukin receptor agonists, TGF- β superfamily receptor agonists, COX-2 inhibitors, MAP kinase inhibitors, inhibitors of matrix metalloproteinases, inhibitors of NF- κ B, nitric oxide synthase inhibitors, agonists and antagonists of integrin receptors, inhibitors of the protein kinase C family, inhibitors of the protein tyrosine kinase family, modulators of protein tyrosine phosphatases and inhibitors of protein src homology 2 domains.

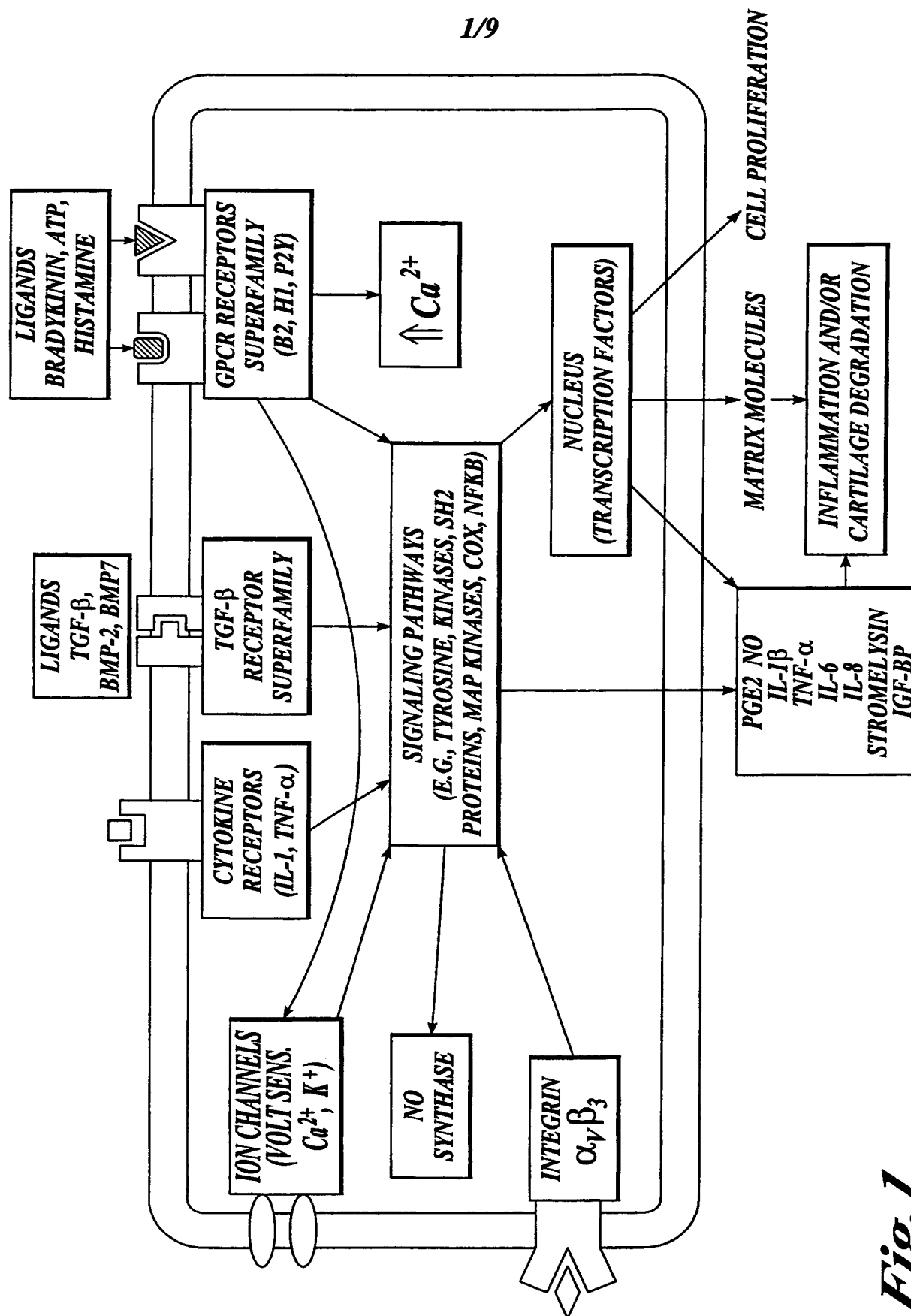
34. The solution of Claim 32, wherein the inhibitor of pain or inflammation is selected from the group consisting of serotonin receptor antagonists, serotonin receptor agonists, histamine receptor antagonists, bradykinin receptor antagonists, kallikrein inhibitors, tachykinin receptor antagonists, calcitonin gene-related peptide (CGRP) receptor antagonists, interleukin receptor antagonists, inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, prostanoid receptor antagonists, leukotriene receptor antagonists, opioid receptor agonists, purinoceptor antagonists, and calcium channel antagonists.

35. A solution for use in the inhibition of cartilage degradation at a joint of a patient, comprising at least one anabolic chondroprotective agent and a second chondroprotective agent that is an inhibitor of cartilage catabolism, the concentration of each agent within the solution being the concentration of that agent which is desired to be delivered locally, in the absence of metabolic transformation, to a joint in order to achieve a predetermined level of cartilage degradation inhibitory effect at the surgical site.

36. The solution of Claim 35, wherein the chondroprotective agents are selected from the group consisting of interleukin-1 receptor antagonists, tumor necrosis factor receptor antagonists, interleukin receptor agonists, TGF- β superfamily receptor agonists, COX-2 inhibitors, MAP kinase inhibitors, inhibitors of matrix metalloproteinases, inhibitors of NF- κ B, nitric oxide synthase inhibitors, agonists and antagonists of integrin receptors, inhibitors of the protein kinase C family, inhibitors of the protein tyrosine kinase family, modulators of protein tyrosine phosphatases and inhibitors of protein src homology 2 domains.

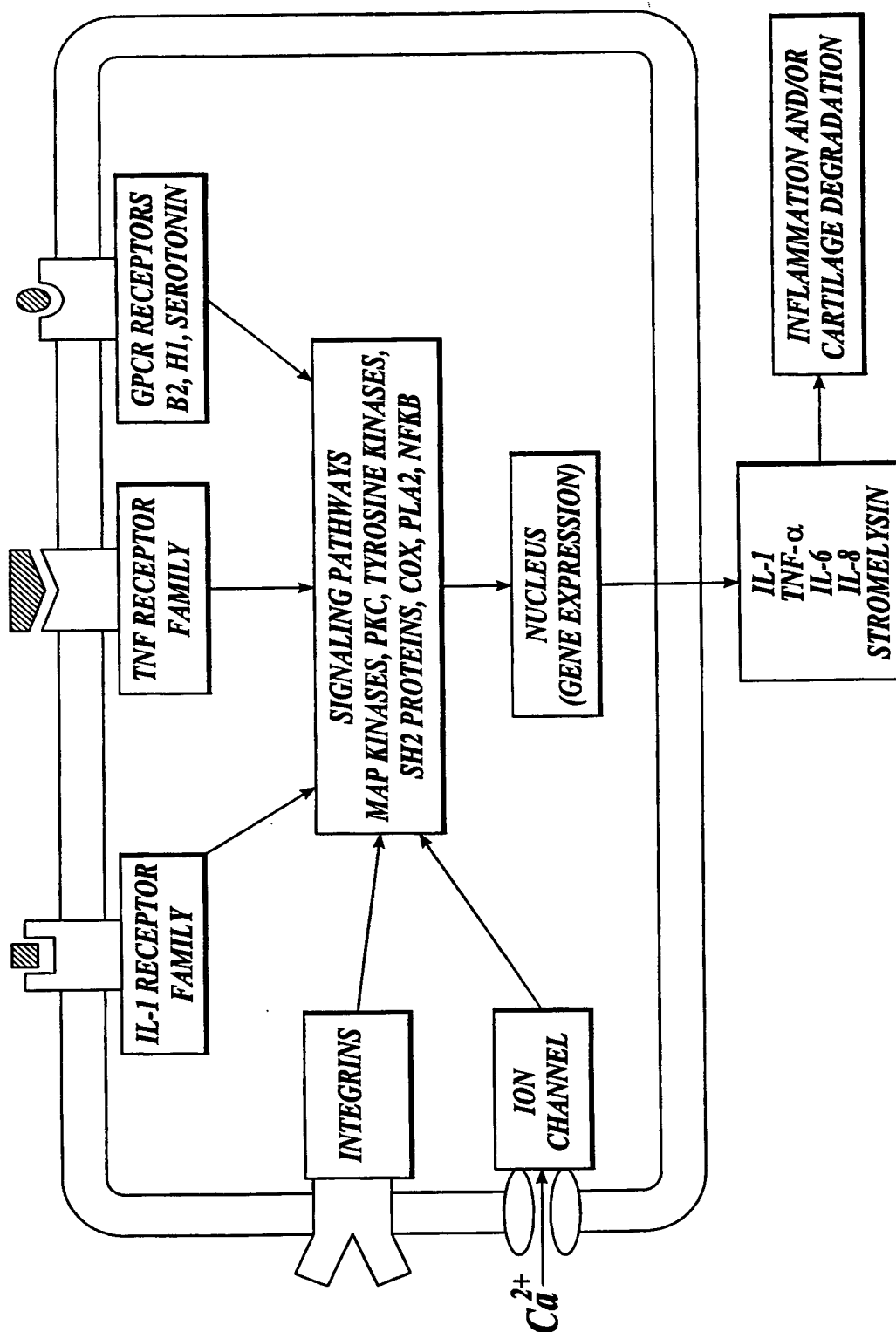
37. The solution of Claim 36, which further comprises at least one inhibitor of pain or inflammation selected from the group consisting of serotonin receptor antagonists, serotonin receptor agonists, histamine receptor antagonists, bradykinin receptor antagonists, kallikrein inhibitors, tachykinin receptor antagonists, calcitonin gene-related peptide (CGRP) receptor antagonists, interleukin receptor antagonists, inhibitors of enzymes active in the synthetic pathway for arachidonic acid metabolites, prostanoid receptor antagonists, leukotriene receptor antagonists, opioid receptor agonists, purinoceptor antagonists, and calcium channel antagonists.

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**Fig. 1**

CHONDROCYTE CELL MOLECULAR TARGETS & SIGNALING INFORMATION

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**Fig. 2**SYNOVIAL CELL MOLECULAR TARGETS & SIGNALING INFORMATION

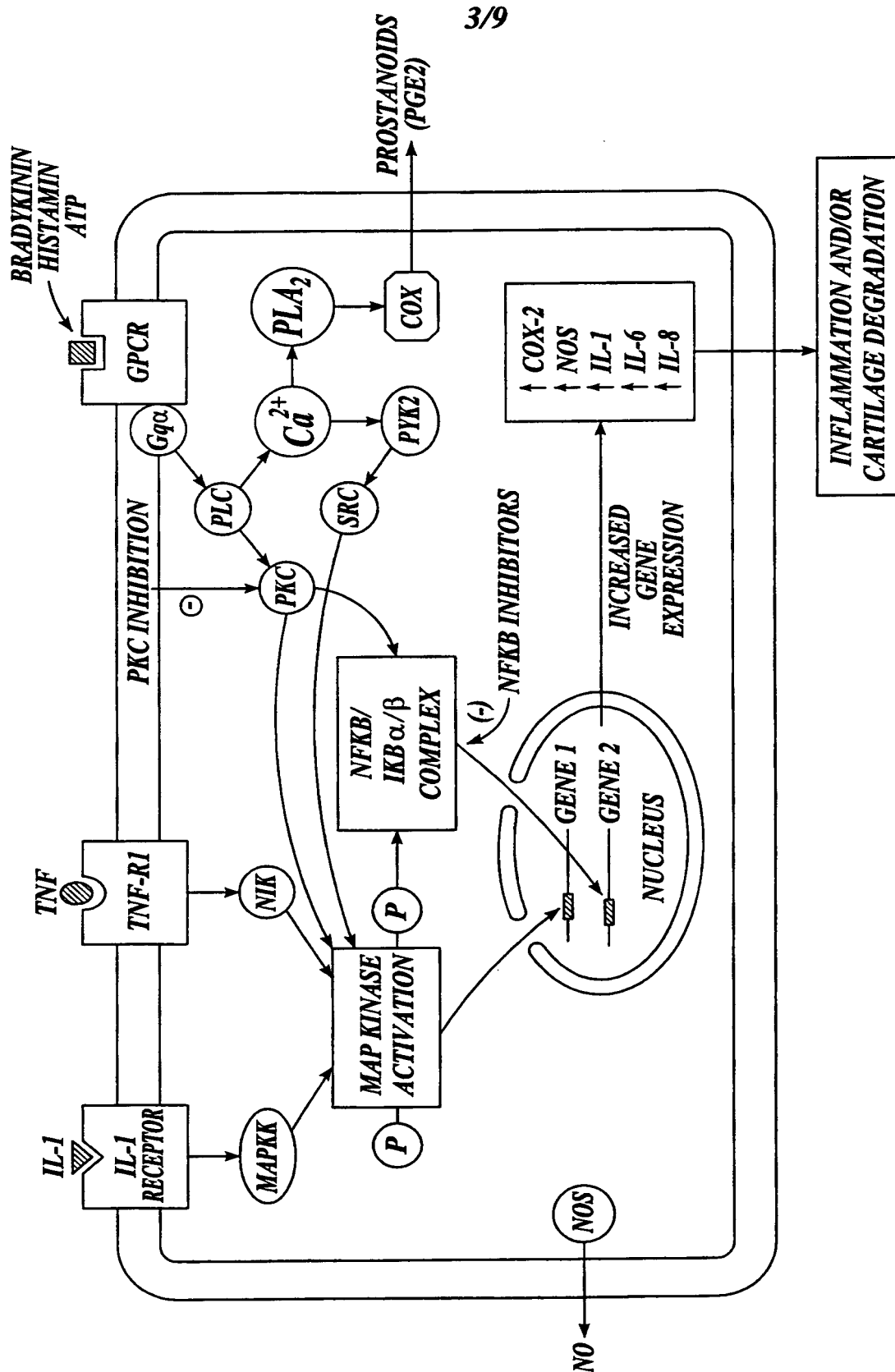


Fig. 3

COMMON SIGNALING PATHWAYS IN CHONDROCYTE/SYNOVIOCYTE CELLS

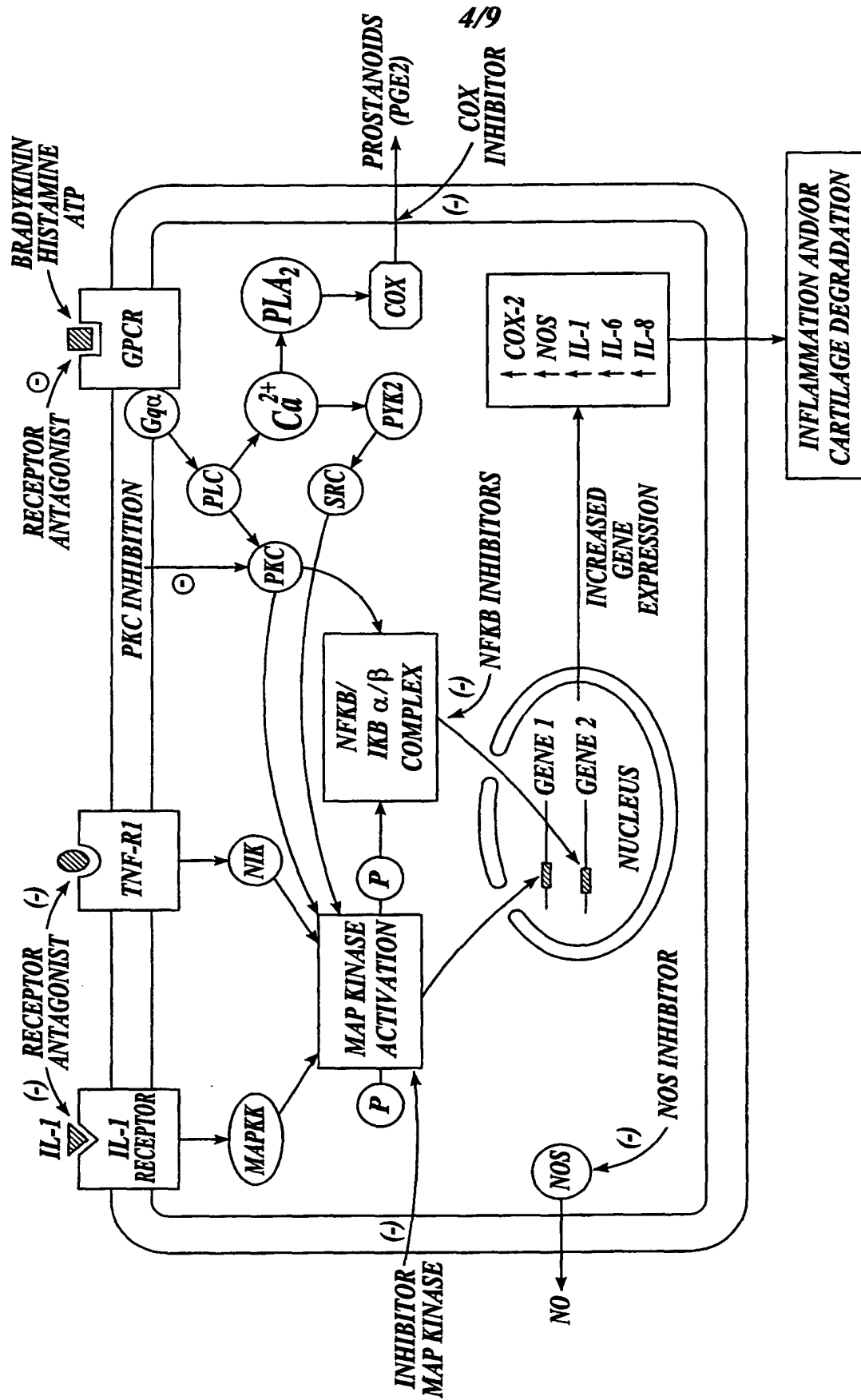


Fig. 4

MOLECULAR SITES OF ACTION IN CHONDROPROTECTIVE SOLUTION

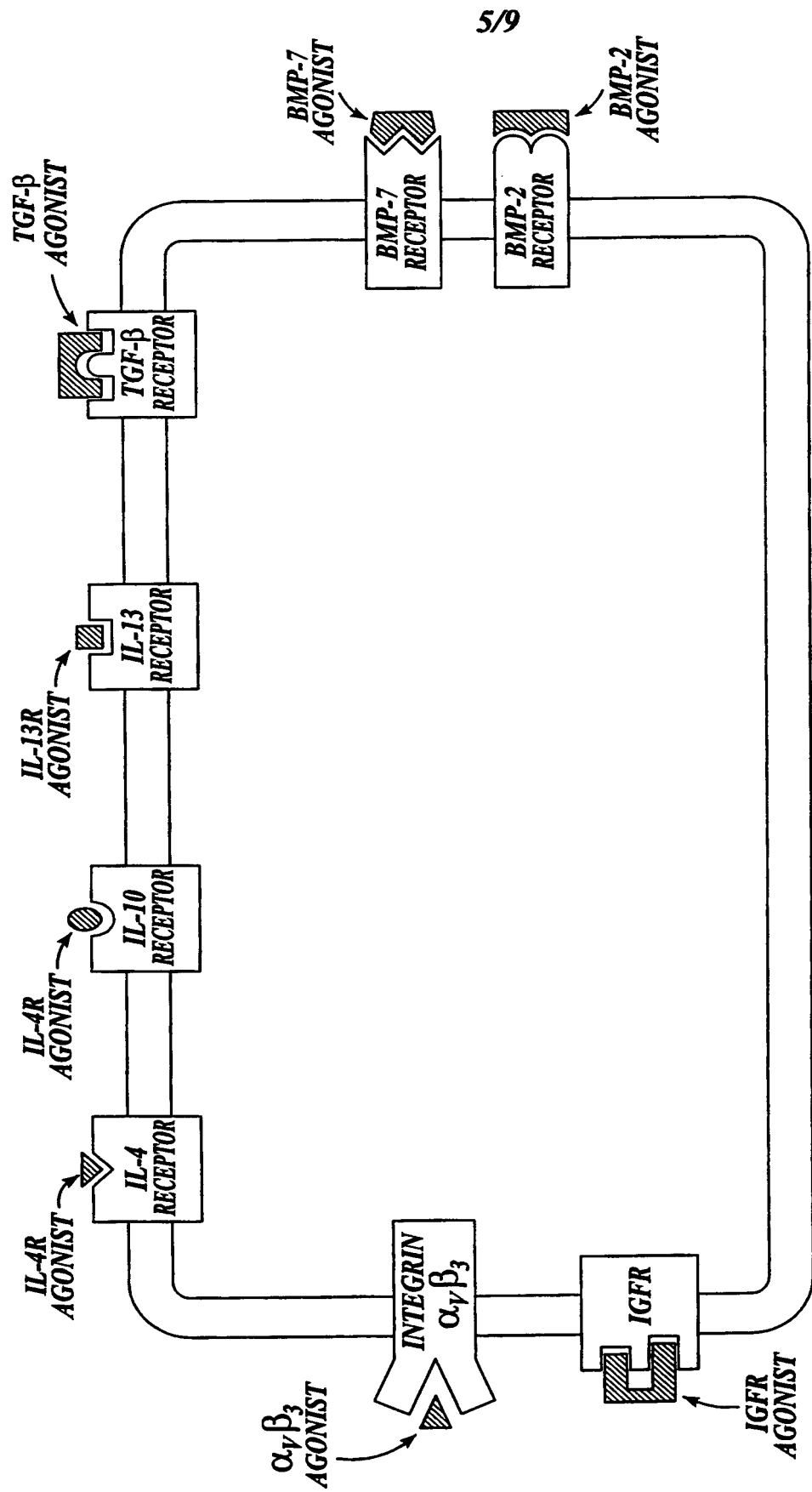


Fig. 5 ANABOLIC CHONDROPROTECTIVE MOLECULAR TARGETS ON SYNOVIOCYTES AND/OR CHONDROCYTES

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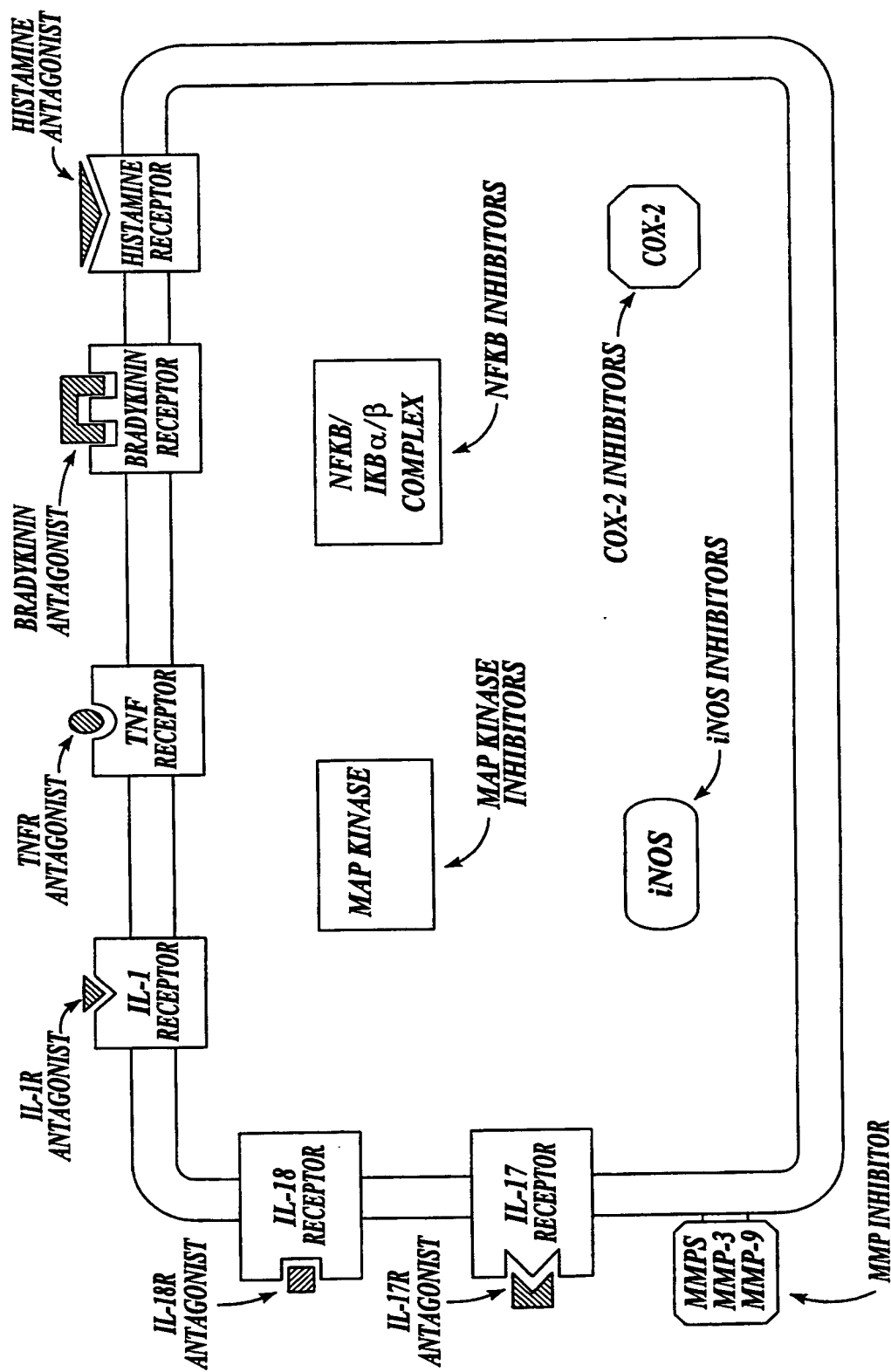


Fig. 6 CATABOLIC CHONDROPROTECTIVE MOLECULAR TARGETS ON SYNOVIOCYTES AND/OR CHONDROCYTES

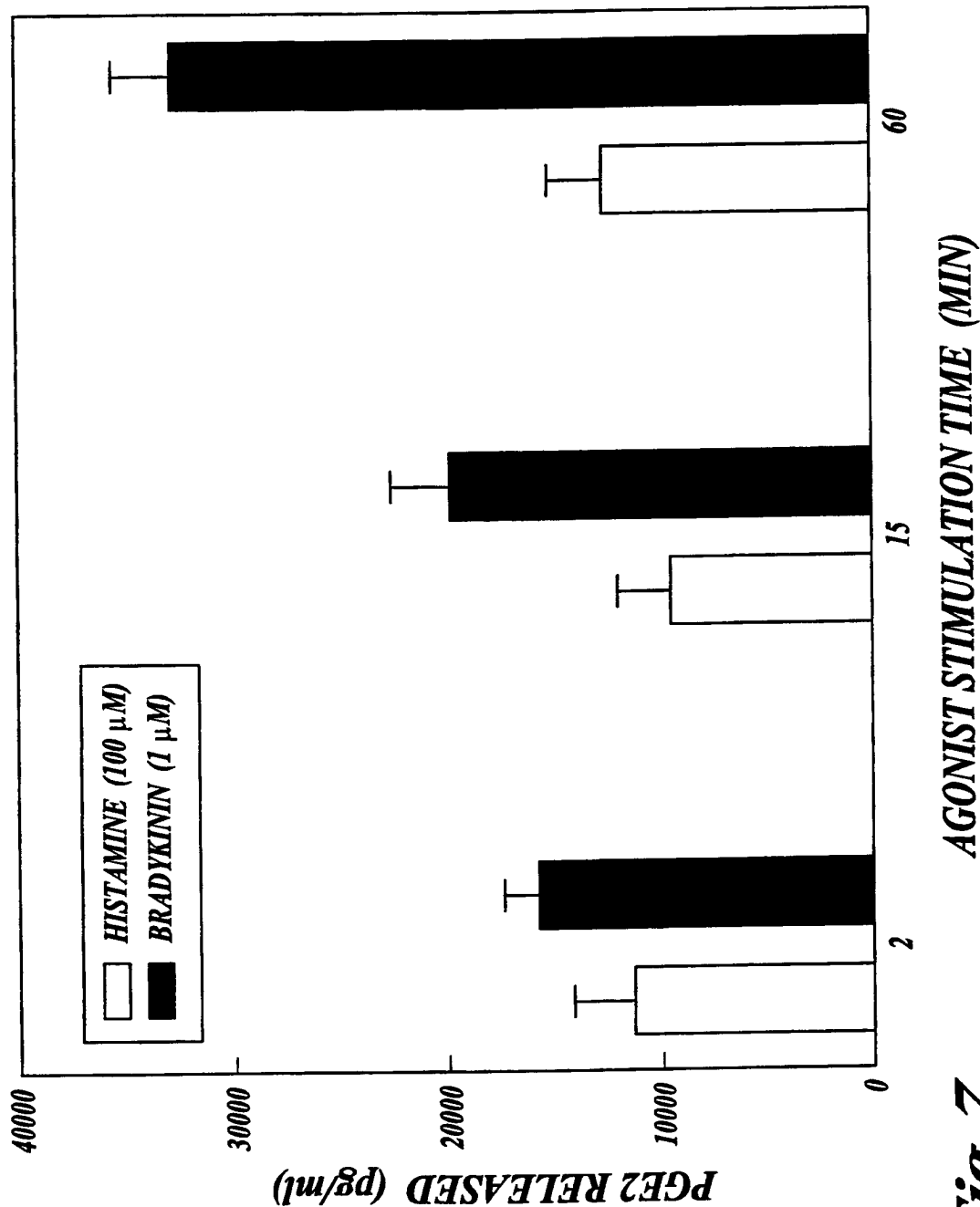
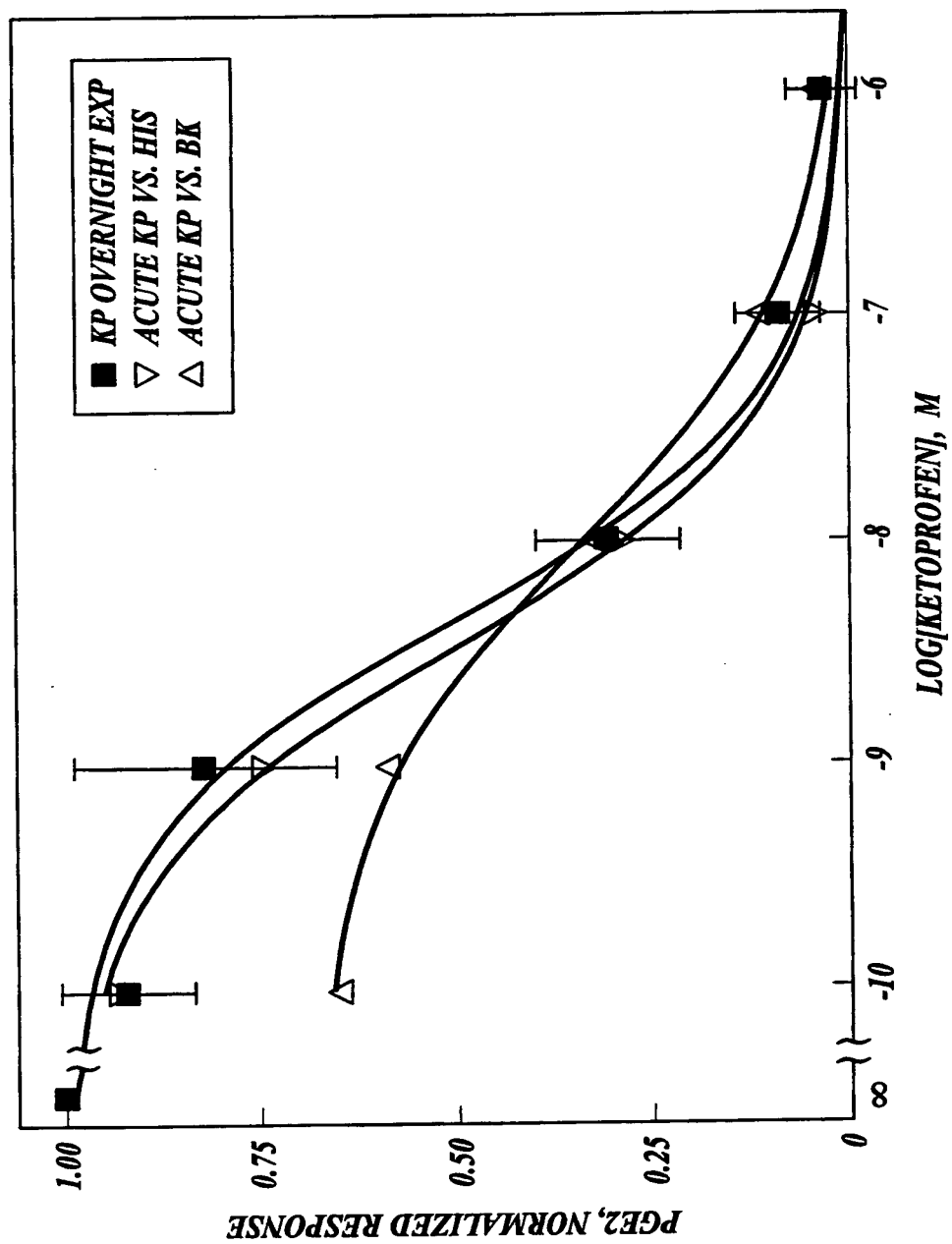
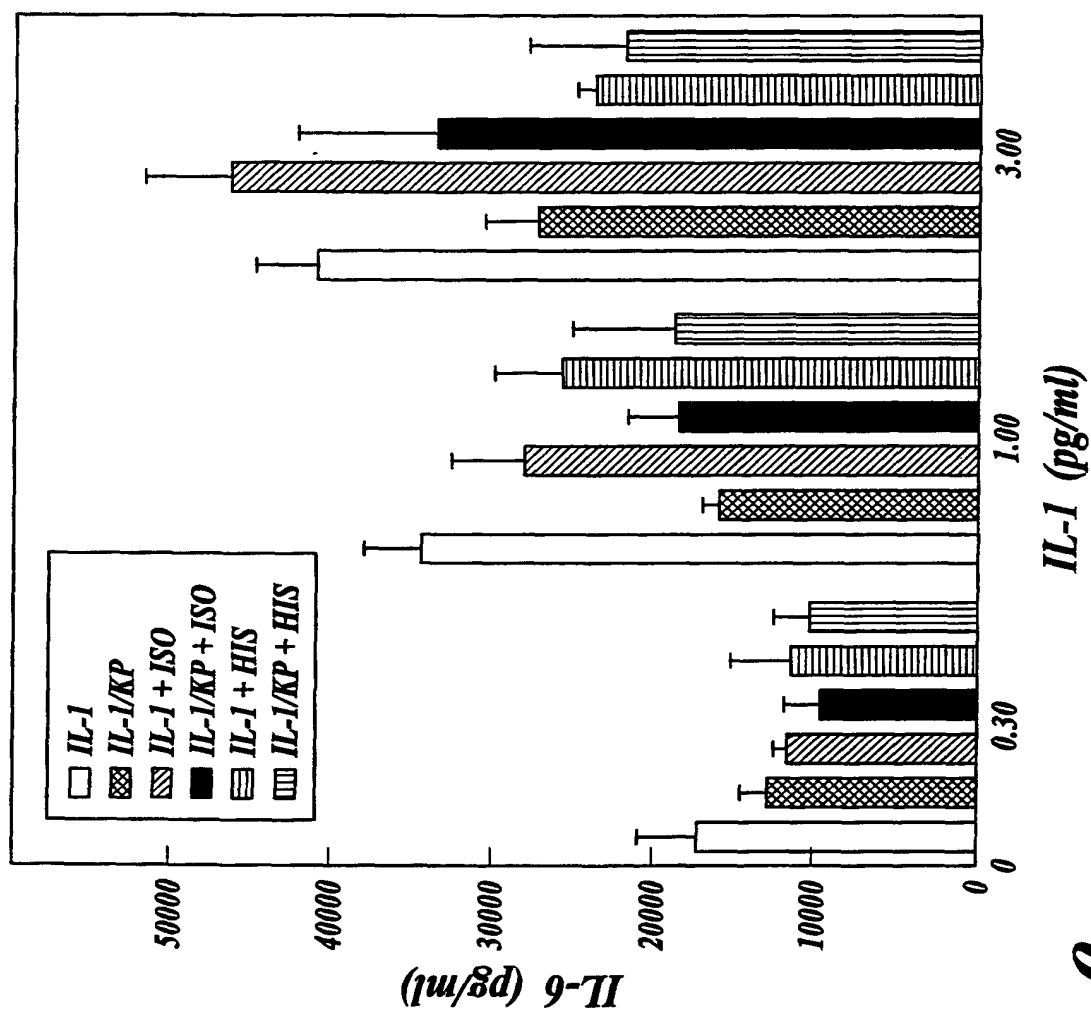


Fig. 7

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*Fig. 8*

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**Fig. 9**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: INHIBITORS OF NITRIC OXIDE SYNTHASE			
(57) Abstract The current invention discloses novel methods for the inhibition of inducible nitric oxide synthesis (iNOS) and the production of NO. Methods of inhibiting the induction of proinflammatory cytokines are also described. Methods of treating various disease states, such as X-linked adrenoleukodystrophy, multiple sclerosis, Alzheimer's and septic shock using inhibitors of iNOS and cytokine induction are disclosed. The inhibitors include the exemplary compounds lovastatin, a sodium salt of phenylacetic acid (NaPA), FPT inhibitor II, N-acetyl cysteine (NAC), and cAMP.			

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DESCRIPTION

INHIBITORS OF NITRIC OXIDE SYNTHASE

BACKGROUND OF THE INVENTION

1. Field of the Invention

5 The present invention relates generally to the treatment of conditions involving undesired or pathological levels of inducible nitric oxide synthase (iNOS), *e.g.* septic shock or neuroinflammatory diseases. In one important aspect, the invention relates to methods of suppressing, inhibiting or preventing the accumulation of nitric-oxide induced cytotoxicity by using inhibitors that block or suppress the induction of cytokines and/or inducible nitric oxide
10 synthase. Another aspect of the invention is the treatment of conditions involving undesired or pathological levels of proinflammatory cytokines (*i.e.* TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ) and/or iNOS. One important aspect of the invention relates to methods of suppressing, inhibiting, or preventing proinflammatory cytokines and/or iNOS induced or aggravated disorders including conditions involving the detrimental effects of inflammation (*e.g.* disorders
15 such as lupus, rheumatoid arthritis, osteoarthritis, amyotrophic lateral sclerosis, and autoimmune disorders; ischemia/reperfusion; neuroinflammatory conditions such as Alzheimer's, stroke, multiple sclerosis, X-linked adrenoleukodystrophy; and the effects of aging).

2. Description of Related Art

Nitric Oxide and Proinflammatory Cytokines

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector
25 molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Nathan, 1992; Jaffrey *et al.*, 1995). NO appears to have both neurotoxic and neuroprotective effects and may have a role in the pathogenesis of stroke and other neurodegenerative diseases and in demyelinating conditions (*e.g.*, multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) and in ischemia and traumatic
30 injuries associated with infiltrating macrophages and the production of proinflammatory

cytokines (Mitrovic *et al.*, 1994; Bo *et al.*, 1994; Merrill *et al.*, 1993; Dawson *et al.*, 1991, Kopranski *et al.*, 1993; Bonfoco *et al.*, 1995). A number of pro-inflammatory cytokines and endotoxin (bacterial lipopolysaccharide, LPS) also induce the expression of iNOS in a number of cells, including macrophages, vascular smooth muscle cells, epithelial cells, fibroblasts, glial
5 cells, cardiac myocytes as well as vascular and non-vascular smooth muscle cells. Although monocytes/macrophages are the primary source of iNOS in inflammation, LPS and other cytokines induce a similar response in astrocytes and microglia (Hu *et al.*, 1995; Galea *et al.*, 1992).

During inflammation, reactive oxygen species (ROS) are generated by various cells
10 including activated phagocytic leukocytes; for example, during the neutrophil "respiratory burst", superoxide anion is generated by the membrane-bound NADPH oxidase. ROS are also believed to accumulate when tissues are subjected to inflammatory conditions including ischemia followed by reperfusion. Superoxide is also produced under physiological conditions and is kept in check by superoxide dismutates. Excessively produced superoxide overwhelms
15 the antioxidant capacity of the cell and reacts with NO to form peroxynitrite, ONOO⁻, which may decay and give rise to hydroxyl radicals, [•]OH (Marietta, M., 1989; Moncada *et al.*, 1989; Saran *et al.*, 1990; Beckman *et al.* 1990). NO, peroxynitrite and [•]OH are potentially toxic molecules to cells including neurons and oligodendrocytes that may mediate toxicity through modification of biomolecules including the formation of iron-NO complexes of iron containing
20 enzyme systems (Drapier *et al.*, 1988), oxidation of protein sulfhydryl groups (Radi *et al.*, 1991), nitration of proteins and nitrosylation of nucleic acids and DNA strand breaks (Wink *et al.*, 1991).

There is now substantial evidence that iNOS plays an important role in the pathogenesis of a variety of diseases. In addition, it is now thought that excess NO production may be
25 involved in a number of conditions, including conditions that involve systemic hypotension such as septic and toxic shock and therapy with certain cytokines. Circulatory shock of various etiologies is associated with profound changes in the body's NO homeostasis. In animal models of endotoxic shock, endotoxin produces an acute release of NO from the constitutive isoform of nitric oxide synthase in the early phase, which is followed by induction of iNOS. NO derived
30 from macrophages, microglia and astrocytes has been implicated in the damage of myelin producing oligodendrocytes in demyelinating disorders like multiple sclerosis and neuronal

death during neuronal degenerating conditions including brain trauma (Hu *et al.*, 1995; Galea *et al.*, 1992; Koprowski *et al.*, 1993; Mitrovic *et al.*, 1994; Bo *et al.*, 1994; Merrill *et al.*, 1993).

NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Nathan, 1992). Nitric oxide synthases are classified into two groups. One type, constitutively expressed (cNOS) in several cell types (*e.g.*, neurons, endothelial cells), is regulated predominantly at the post-transcriptional level by calmodulin in a calcium dependent manner (Nathan, 1992; Jaffrey *et al.*, 1995). In contrast, the inducible form (iNOS), synthesized *de novo* in response to different stimuli in various cell types including macrophages, hepatocytes, myocytes, neutrophils, endothelial and mesangial cells, is independent of calcium. Astrocytes, the predominant glial component of brain have also been shown to induce iNOS in response to bacterial lipopolysaccharide (LPS) and a series of proinflammatory cytokines including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ) (Hu *et al.*, 1995; Galea *et al.*, 1992).

Cytokines associated with extracellular signaling are involved in the normal process of host defense against infections and injury, in mechanisms of autoimmunity and in the pathogenesis of chronic inflammatory diseases. It is believed that nitric oxide (NO), synthesized by nitric oxide synthetase (NOS) mediates deleterious effects of the cytokines (Nathan, 1987; Zang *et al.*, 1993; Kubes *et al.*, 1991). For example, NO as a result of stimuli by cytokines (*e.g.*, TNF- α , IL-1 and interleukin-6 (IL-6)) is implicated in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, osteoarthritis (Zang *et al.*, 1993; McCartney-Francis *et al.*, 1993). The NO produced by iNOS is associated with bactericidal properties of macrophages (Nathan, 1992; Stuehr *et al.*, 1989). Recently, an increasing number of cells (including muscle cells, macrophages, keratinocytes, hepatocytes and brain cells) have been shown to induce iNOS in response to a series of proinflammatory cytokines including IL-1, TNF- α , interferon- γ (IFN- γ) and bacterial lipopolysaccharides (LPS) (Zang *et al.*, 1993; Busse *et al.*, 1990; Genge *et al.*, 1995).

Signal Transduction Pathways

Mevalonate metabolites, particularly farnesyl pyrophosphate (FPP), are involved in post-translational modification of some G-proteins, including Ras (Goldstein *et al.*, 1990;

Casey *et al.*, 1989). The inhibition of isoprenylation of Ras proteins by inhibitors of mevalonate pathway and their membrane association and transduction of signal from Ras to Raf/MAP kinase cascade (Kikuchi *et al.*, 1994) indicates a role of mevalonate metabolites in the transduction of signal from receptor tyrosine kinases to Raf/MAP kinase cascade. Two enzymes that control the rate-limiting steps of the mevalonate pathway are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the formation of mevalonate from acetyl-CoA, and mevalonate pyrophosphate decarboxylase, which controls the use of mevalonate within the cell by converting 3-phospho-5-pyrophospho-mevalonate to isopentenyl pyrophosphate. Lovastatin, a potent inhibitor of HMG-CoA reductase, and sodium salt of phenylacetic acid (NaPA), an inhibitor of mevalonate pyrophosphate decarboxylase, are known to reduce the level of cellular isoprenoids (Castillo *et al.*, 1991; Samid *et al.*, 1994) and isoprenylated proteins (Repko and Maltese, 1989). No suppression of isoprenylated protein maturation *in vitro* by lovastatin treatment that produced 50% inhibition of sterol biosynthesis has been observed (Sinensky *et al.*, 1991). The IC_{50} for inhibition of sterol synthesis is 10 nM, whereas the IC_{50} for inhibition of conversion of pro-p21^{ras} to mature-p21^{ras} is maximal at 2.6 μ M (Sinensky *et al.*, 1991). The pharmacologically attainable concentration for NaPA, however, is 1 to 5 mM (Thibault *et al.*, 1995). HMG-CoA reductase can also be inhibited by 5-amino 4-imidazolecarboxamide ribotide (AICAR). AICAR stimulates AMP-activated protein kinase, an enzyme that inhibits acetyl-CoA carboxylase and HMG-CoA reductase (Henin *et al.*, 1995)

LPS is shown to bind cell-surface receptor CD14 (Stefanova *et al.*, 1993) and induce iNOS, probably via activation of NF κ B (Xie *et al.*, 1994; Kwon *et al.*, 1995). NF κ B is an ubiquitous multisubunit transcription factor that is activated in response to various stimuli including cytokines TNF- α , IL-1, IL-2, IL-6, viruses, LPS, DNA damaging agents and phorbol myristate acetate (PMA) (Schreck *et al.*, 1992). Previous studies (Law *et al.*, 1992) demonstrating the inhibition of NF- κ B activation by mevinolin and 5'-methylthioadenosine indicates a role of protein farnesylation and carboxyl methylation reactions in the activation of NF- κ B. Identification of the binding site of NF- κ B in the promoter region of the iNOS gene and that the activation of NF κ B in LPS-induced iNOS induction establishes a role of NF κ B activation in the induction of iNOS (Xie *et al.*, 1994). Although the precise mechanism of NF κ B activation is not known at the present time, the inhibition of activation of NF κ B by

inhibitors of tyrosine kinase and proteases indicates a role of phosphorylation and degradation of I κ B in this process (Menon *et al.*, 1993; Henkel *et al.*, 1993).

Reactive oxygen (Schreck *et al.*, 1992) and reactive nitrogen (Lander *et al.*, 1993) species have been demonstrated to mediate the signal for NF κ B activation. The differential induction of NF κ B by protein phosphatase inhibitors in primary and transformed cell lines also indicates that induction of NF κ B is dependent on the dual processes of cellular redox and phosphorylation (Menon *et al.*, 1993). The exact target of ROS that modulate cellular redox is unknown, and the lack of induction in cells in which activity of p21ras was blocked through expression of a dominant negative mutant or treatment with farnesyltransferase inhibitor indicate that direct activation of p21ras may be the central mechanism by which redox stress stimuli transmit its signal to the nucleus (Lander *et al.*, 1995).

Ceramide Production and Apoptosis

Cytokine-mediated ceramide production is implicated in apoptosis of different cells including brain cells (Brugg *et al.*, 1996; Wiesner and Dawson, 1996). Several studies support a role for hydrolysis of sphingomyelin as a stress-activated signaling mechanism in which ceramide plays a role in cell regulation, cell differentiation, growth suppression and apoptosis in various cell types including glial and neuronal cells (Hannun and Bell, 1989; Hannun, 1994; Kolesnick and Golde, 1994; Brugg *et al.*, 1996; Wiesner and Dawson, 1996). Sphingomyelin is preferentially concentrated in the outer leaflet of the plasma membrane of most mammalian cells; it comprises sphingosine (a long chain sphingoid base backbone), a fatty acid, and a phosphocholine head group. Ceramide is composed of a sphingoid base with a fatty acid in amide linkage. Ceramide activates the proteases of the interleukin-converting enzyme (ICE) family (especially prICE/YAMA/ CPP32), the protease responsible for cleavage of poly(A)DP-ribose polymerase (Martin *et al.*, 1995), and that the activation of prICE by ceramide and induction of apoptosis are inhibited by overexpression of Bcl-2 (Zhang *et al.*, 1996). Addition of exogenous ceramides or sphingomyelinase to cells induces stress-activated protein kinase-dependent transcriptional activity through the activation of c-jun (Latinis and Koretzky, 1996), and a dominant negative mutant of SEK1, the protein kinase responsible for phosphorylation and activation of stress-activated protein kinase, interferes with ceramide-induced apoptosis (Verheij *et al.*, 1996). These observations also indicate that both

Bcl-2 and stress-activated protein kinase function downstream of ceramide in the apoptotic pathway.

The signaling events in cytokine-mediated activation of sphingomyelin degradation to ceramide are poorly understood. Since the discovery of the sphingomyelin cycle, several
5 inducers have been shown to be coupled to sphingomyelin-ceramide signaling events, including $1\alpha,25$ -dihydroxyvitamin D_3 , radiation, antibody cross-linking, TNF- α , IFN- γ , IL-1 β , nerve growth factor, and brefeldin A (Hannun and Bell, 1989; Hannun, 1994; Kolesnick and Golde, 1994; Zhang and Kolesnick, 1995; Kantey *et al.*, 1995; Linardic *et al.*, 1996).

The sphingomyelin pathway-associated signal transduction pathway mediates the action
10 of several extracellular stimuli that lead to important biochemical and cellular effects (Zhang and Kolesnick, 1995; Kantey *et al.*, 1995; Yao *et al.*, 1995; Hannun, 1996; Lozano *et al.*, 1994). In the case of TNF- α , the pathway is initiated by the action of TNF- α on its 55-kDa receptor, leading to phospholipase A_2 activation, generation of arachidonic acid, and subsequent activation of sphingomyelinase (Jayadev *et al.*, 1994). This pathway is initiated by the
15 activation of two distinct forms of sphingomyelinase (SMase), a membrane-associated neutral sphingomyelinase (Chatterjee, 1993) and an acidic sphingomyelinase (Spence, 1993), which reside in the caveola and the endosomal-lysosomal compartment. Each type of SMase hydrolyzes the phosphodiester bond of sphingomyelin to yield ceramide and phosphocholine. Proinflammatory cytokines (tumor necrosis factor- α , TNF- α ; interleukin-1 β , IL-1 β ;
20 interferon- γ , IFN- γ) and bacterial lipopolysaccharides have been shown as potent inducers of SMases. Ceramide has emerged as a second messenger molecule that is considered to mimic most of the cellular effects of cytokines and lipopolysaccharide in terminal differentiation, apoptosis, and cell cycle arrest (Zhang and Kolesnick, 1995; Kantey *et al.*, 1995).

Sphingomyelin turnover and ceramide generation in response to TNF- α and IL-1 β
25 occurs within minutes of stimulation; however, the sequence of events linking receptor stimulation and SMase activation remains largely unknown (Hannun, 1996; Lozano *et al.*, 1994; Jayadev *et al.*, 1994). In a number of cell systems, interaction of TNF- α with its membrane receptors (p75 and p55) has been found to activate phospholipase A_2 and to induce release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine pools. This
30 arachidonic acid has been shown as a mediator of sphingomyelin hydrolysis in response to TNF- α (Jayadev *et al.*, 1994). In addition, proteases have also been implicated in the pathway

leading from TNF- α to the activation of SMase (Hannun, 1996; Dbaio *et al.*, 1997) recently. On the other hand, IL-1 β and TNF- α are known to induce the production of reactive oxygen species, a class of highly diffusible and ubiquitous molecules, which have been suggested to act as second messengers (Tiku *et al.*, 1990; Lo and Cruz, 1995; Devary *et al.*, 1991). ROS encompassing species such as superoxide, hydrogen peroxide, and hydroxyl radicals are known to regulate critical steps in the signal transduction cascade and many important cellular events including protein phosphorylation, gene expression, transcription factor activation, DNA synthesis, and cellular proliferation (Schreck *et al.*, 1991; Sen and Packer, 1996). A recent observation has shown that glutathione or similar molecules inhibit the activity of magnesium-dependent neutral SMase *in vitro* (Liu and Hannun, 1997). However, surprisingly, the SH group of GSH was not required as S-methyl GSH and GSSG inhibited neutral SMase at lower concentrations than GSH (Liu and Hannun, 1997). On the other hand, N-acetylcysteine has also been found to inhibit the synthesis of ceramide in cultured rat hepatocytes through the inhibition of dihydroceramide desaturase (Michel *et al.*, 1997).

Inflammatory Diseases

NO generated by iNOS has been implicated in the pathogenesis of inflammatory diseases. In experimental animals hypotension induced by LPS or TNF- α can be reversed by NOS inhibitors and reinitiated by L-arginine (Kilbourn *et al.*, 1990). Conditions which lead to cytokine-induced hypotension include septic shock, hemodialysis (Beasley and Brenner, 1992) and IL-2 therapy in cancer patients (Hibbs *et al.*, 1992). Studies in animal models have suggested a role for NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease (Miller *et al.*, 1990) and cerebral ischemia and arthritis (Ialenti *et al.*, 1993; Stevanovic-Racic *et al.*, 1994).

Inflammation, iNOS activity and/or cytokine production has been implicated in a variety of diseases and conditions, including psoriasis (Ruzicka *et al.*, 1994; Kolb-Bachofen *et al.*, 1994; Bull *et al.*, 1994); uveitis (Mandia *et al.*, 1994); type 1 diabetes (Eisieik & Leijersfam, 1994; Kroncke *et al.*, 1991; Welsh *et al.*, 1991); septic shock (Petros *et al.*, 1991; Thiernemann & Vane, 1992; Evans *et al.*, 1992; Schilling *et al.*, 1993); pain (Moore *et al.*, 1991; Moore *et al.*, 1992; Meller *et al.*, 1992; Lee *et al.*, 1992); migraine (Olesen *et al.*, 1994); rheumatoid arthritis (Kaur & Halliwell, 1994); osteoarthritis (Stadler *et al.*, 1991);

inflammatory bowel disease (Miller *et al.*, 1993; Miller *et al.*, 1993); asthma (Hamid *et al.*, 1993; Kharitonov *et al.*, 1994); Koprowski *et al.*, 1993); immune complex diseases (Mulligan *et al.*, 1992); multiple sclerosis (Koprowski *et al.*, 1993); ischemic brain edema (Nagafuji *et al.*, 1992; Buisson *et al.*, 1992; Trifiletti *et al.*, 1992); toxic shock syndrome
5 (Zembowicz & Vane, 1992); heart failure (Winlaw *et al.*, 1994); ulcerative colitis (Boughton-Smith *et al.*, 1993); atherosclerosis (White *et al.*, 1994); glomerulonephritis (Muhl *et al.*, 1994); Paget's disease and osteoporosis (Lowick *et al.*, 1994); inflammatory sequelae of viral infections (Koprowski *et al.*, 1993); retinitis, (Goureau *et al.*, 1992); oxidant induced lung injury (Berisha *et al.*, 1994); eczema (Ruzica *et al.*, 1994); acute allograft rejection (Devlin,
10 J. *et al.*, 1994); and infection caused by invasive microorganisms which produce NO (Chen, Y and Rosazza, J. P. N., 1994).

In the central nervous system, apoptosis may play an important pathogenetic role in neurodegenerative diseases such as iscehmic injury and white matter diseases (Thompson, 1995; Bredesen, 1995). Both X-linked adrenoleukodystrophy (X-ALD) and multiple sclerosis
15 (MS) are demyelinating diseases with the involvement of proinflammatory cytokines in the manifestation of white matter inflammation. The presence of immunoreactive tumor necrosis factor a (TNF- α) and interleukin 1 (IL-1 β) in astrocytes and microglia of X-ALD brain has indicated the involvement of these cytokines in immunopathology of X-ALD and aligned
X-ALD with MS, the most common immune-mediated demyelinating disease of the CNS in
20 man (Powers, 1995; Powers *et al.*, 1992; McGuinness *et al.*, 1995; McGuinness *et al.*, 1997). Several studies demonstrating the induction of proinflammatory cytokines at the protein or mRNA level in cerebrospinal fluid and brain tissue of MS patients have established an association of proinflammatory cytokines (TNF- α , IL-1 β , IL-2, IL-6, and IFN- γ) with the
inflammatory loci in MS (Maimone *et al.*, 1991; Tsukada *et al.*, 1991; Rudick and Ransohoff,
25 1992).

X-linked adrenoleukodystrophy (X-ALD), an inherited, recessive peroxisomal disorder, is characterized by progressive demyelination and adrenal insufficiency (Singh, 1997; Moser *et al.*, 1984). It is the most common peroxisomal disorder affecting between 1/15,000 to 1/20,000 boys and manifests with different degrees of neurological disability. The onset of
30 childhood X-ALD, the major form of X-ALD, is between the age of 4 to 8 and then death within the next 2 to 3 years. Although X-ALD presents as various clinical phenotypes,

including childhood X-ALD, adrenomyeloneuropathy (AMN), and Addison's disease, all forms of X-ALD are associated with the pathognomonic accumulation of saturated very long chain fatty acids (VLCFA) (those with more than 22 carbon atoms) as a constituent of cholesterol esters, phospholipids and gangliosides (Moser *et al.*, 1984) and secondary neuroinflammatory damage (Moser *et al.*, 1995). The neurologic damage in X-linked adrenoleukodystrophy may be mediated by the activation of astrocytes and the induction of proinflammatory cytokines. Due to the presence of similar concentration of VLCFA in plasma and as well as in fibroblasts of X-ALD, fibroblasts are generally used for both prenatal and postnatal diagnosis of the disease (Singh, 1997; Moser *et al.*, 1984).

The deficient activity for oxidation of lignoceroyl-CoA ligase as compared to the normal oxidation of lignoceroyl-CoA in purified peroxisomes isolated from fibroblasts of X-ALD indicated that the abnormality in the oxidation of VLCFA may be due to deficient activity of lignoceroyl-CoA ligase required for the activation of lignoceric acid to lignoceroyl-CoA (Hashmi *et al.*, 1986; Lazo *et al.*, 1988). While these metabolic studies indicated lignoceroyl-CoA ligase gene as a X-ALD gene, positional cloning studies led to the identification of a gene that encodes a protein (ALDP), with significant homology with the ATP-binding cassette (ABC) of the super-family of transporters (Mosser *et al.*, 1993). The normalization of fatty acids in X-ALD cells following transfection of the X-ALD gene (Cartier *et al.*, 1995) supports a role for ALDP in fatty acid metabolism; however, the precise function of ALDP in the metabolism of VLCFA is not known at present.

Similar to other genetic diseases affecting the central nervous system, the gene therapy in X-ALD does not seem to be a real option in the near future and in the absence of such a treatment a number of therapeutic applications have been investigated (Singh, 1997; Moser, 1995). Adrenal insufficiency associated with X-ALD responds readily with steroid replacement therapy, however, there is as yet no proven therapy for neurological disability (Moser, 1995). Addition of monoenoic fatty acid (*e.g.*, oleic acid) to cultured skin fibroblasts of X-ALD patients causes a reduction of saturated VLCFA presumably by competition for the same chain elongation enzyme (Moser, 1995). Treatment of X-ALD patients with trioleate resulted in 50% reduction of VLCFA. Subsequent treatment of X-ALD patients with a mixture of trioleate and trieruciate (popularly known as Lorenzo's oil) also led to a decrease in plasma levels of VLCFA (Moser, 1995; Rizzo *et al.*, 1986; Rizzo *et al.*, 1989). Unfortunately, the clinical efficacy has

been unsatisfactory since no proof of favorable effects has been observed by attenuation of the myelinolytic inflammation in X-ALD patients (Moser, 1995). Moreover, the exogenous addition of unsaturated VLCFA induces the production of superoxide, a highly reactive oxygen radical, by human neutrophils (Hardy *et al.*, 1994). Since cerebral demyelination of X-ALD is associated with a large infiltration of phagocytic cells to the site of the lesion (Powers *et al.*, 1992), treatment with unsaturated fatty acids may even be toxic to X-ALD patients. Bone marrow therapy also appears to be of only limited value because of the complexity of the protocol and of insignificant efficacy in improving the clinical status of the patient (Moser, 1995).

Experimental allergic encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) that serves as a model for the human demyelinating disease, multiple sclerosis (MS). Studies have shown that the majority of the inflammatory cells constitute of T-lymphocytes and macrophages (Merrill and Benveniste, 1996). These effector cells and astrocytes have been implicated in the disease pathogenesis by secreting number of molecules that act as inflammatory mediators and/or tissue damaging agents such as nitric oxide (NO). NO is a molecule with beneficial as well as detrimental effects. In neuroinflammatory diseases like EAE, high amounts of NO produced for longer durations by inducible nitric oxide synthase (iNOS) acts as a cytotoxic agent towards neuronal cells. Previous studies have shown NO by itself or its reactive product (ONOO⁻) may be responsible for death of oligodendrocytes, the myelin producing cells of the CNS, and resulting in demyelination in the neuroinflammatory disease processes (Merrill *et al.*, 1993; Mitrovic *et al.*, 1994).

Infiltrating T-lymphocytes in EAE produce pro-inflammatory cytokines such as IL-12, TNF- α and IFN- γ (Merrill and Benveniste, 1996). In addition to T-cells and macrophages, astrocytes have also been shown to produce TNF- α (Shafer and Murphy, 1997). Convincing evidence exists to support a role for both TNF- α and IFN- γ in the pathogenesis of EAE (Taupin *et al.*, 1997; Villarroya *et al.*, 1996; Issazadeh *et al.*, 1995). Investigations with antibodies against TNF- α have shown that in mice these antibodies protect against active and adaptively transferred EAE disease (Klinkert *et al.*, 1997). The expression of TNF- α and IFN- γ during EAE disease could result in the upregulation of iNOS in macrophage and astrocytes because TNF- α and IFN- γ have been shown to be potent inducers of iNOS in macrophages and

astrocytes in culture (Xie *et al.*, 1994). This induction of iNOS could result in the production of NO, which if produced in large amounts may lead to cytotoxic effects. Peroxynitrite (ONOO⁻) has been identified in both MS and EAE CNS (Hooper *et al.*, 1997; van der Veen *et al.*, 1997). The role of peroxynitrite in the pathogenesis of EAE is supported by the beneficial effects of uric acid, a peroxynitrite scavenger, against EAE and by a subsequent survey documenting that MS patients had significantly lower serum uric acid levels than those of controls (Hooper *et al.*, 1998). However, aggravation of EAE by inhibitors of NOS activity (Ruuls *et al.*, 1996) and in an animal model of iNOS gene knockout (Fenyk-Melody *et al.*, 1998) indicate that NO may not be the only pathological mediator in EAE disease process. In addition to NO other free radicals such as reactive oxygen intermediates (O₂⁻, H₂O₂, and OH⁻) can also be stimulated by cytokines (Merrill and Benveniste, 1996). Reactive oxygen intermediates (ROI) and NO are believed to be key mediators of pathophysiological changes that take place during inflammatory disease process. ROI's such as superoxide anion, hydroxy radicals and hydrogen peroxide can also be stimulated by TNF- α (Merrill and Benveniste, 1996). Therefore, it is likely that both the direct modulation of cellular functions by proinflammatory cytokines and toxicity of the ROI and reactive nitrogen species may play a role in the pathogenesis of EAE disease.

Several studies on protein and/or mRNA levels in plasma, cerebrospinal fluid (CSF), brain tissue, and cultured blood leukocytes from MS patients have established an association of proinflammatory cytokines (TNF- α , IL-1 and IFN- γ) with MS (Taupin *et al.*, 1997; Villarroya *et al.*, 1996; Issazadeh *et al.*, 1995). The mRNA for iNOS has also been detectable in both MS as well as EAE brains (Bagasra *et al.*, 1995; Koprowski *et al.*, 1993). Semiquantitative RT-PCRTM for iNOS mRNA in MS brains shows markedly higher expression of iNOS mRNA in MS brains than control brains (Bagasra *et al.*, 1995). Analysis of CSF from MS patients has also shown increased levels of nitrite and nitrate compared with normal control (Merrill and Benveniste, 1996). Peroxynitrite, ONOO⁻ is a strong nitrosating agent capable of nitrosating tyrosine residues of proteins to nitrotyrosine. Increased levels of nitrotyrosine have been found in demyelinating lesions of MS brains as well as spinal cords of mice with EAE (Hooper *et al.*, 1998; Hooper *et al.*, 1997). A strong correlation exists between CSF levels of cytokines, disruption of blood-brain barrier, and high levels of circulating cytokines in MS patients (Villarroya *et al.*, 1996; Issazadeh *et al.*, 1995). Increase in TNF- α and IFN- γ levels seems to predict relapse in MS and the number of circulating IFN- γ positive blood cells correlates with

severity of disability. These observations support the view that in both MS and EAE, induction of proinflammatory cytokines and production of NO through iNOS play roles in the pathogenesis of these diseases.

Alzheimer's disease (AD) is the most common degenerative dementia affecting primarily the elderly population. The disease is characterized by the decline of multiple cognitive functions and a progressive loss of neurons in the central nervous system. Deposition of beta-amyloid peptide has also been associated with AD. Over the last decade, a number of investigators have noted that AD brains contain many of the classical markers of immune mediated damage. These include elevated numbers of microglia cells, which are believed to be an endogenous CNS form of the peripheral macrophage, and astrocytes. Of particular importance, complement proteins have been immunohistochemically detected in the AD brain and they most often appear associated with beta-amyloid containing pathological structures known as senile plaques (Rogers *et al.*, 1992; Haga *et al.*, 1993).

These initial observations which suggest the existence of an inflammatory component in the neurodegeneration observed in AD has been extended to the clinic. A small clinical study using the nonsteroidal anti-inflammatory drug, indomethacin, indicated that indomethacin significantly slowed the progression of the disease (Neurology, 43(8):1609 (1993)). In addition, a study examining age of onset among 50 elderly twin pairs with onsets of AD separated by three or more years, suggested that anti-inflammatory drugs may prevent or delay the initial onset of AD symptoms (Neurology, 44:227 (1994)).

Over the years numerous therapies have been tested for the possible beneficial effects against EAE or MS disease but with mixed results (Cross *et al.*, 1994; Ruuls *et al.*, 1996). Though aminoguanidine (AG) has been described as a competitive inhibitor of iNOS and a suppressor of its expression (Corbett and McDaniel, 1996; Joshi *et al.*, 1996), to date few compounds which inhibit iNOS are of potential therapeutic value have been identified. This deficiency is particularly troubling given the significant cellular damage which can arise as a result of iNOS-mediated nitric oxide toxicity, especially in chronic inflammatory disease states. There is a present need for therapeutic agents which will inhibit or even prevent cytotoxic concentrations of NO from occurring in individuals suffering from diseases and conditions to which NO toxicity or an undesired production of proinflammatory cytokines is linked.

SUMMARY OF THE INVENTION

The invention generally provides methods of treating nitric oxide (NO) cytotoxicity comprising providing a biologically effective amount of an inducible nitric oxide synthase (iNOS) and/or proinflammatory cytokine induction suppressor and/or inhibitor. The invention provides a solution to the cytotoxicity induced or fostered by the presence of NO and/or proinflammatory cytokines which is observed in individuals suffering from autoimmune or inflammatory diseases, including stroke, neurodegenerative diseases, demyelinating conditions (e.g., multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy), brain trauma, ischemia-reperfusion, Alzheimer's disease, aging, Landry-Guillain-Barre-Strohl syndrome, rheumatoid arthritis, endotoxic shock, myocardial infarction, tissue injury or HIV-mediated NO neurotoxicity.

The invention first provides a method for suppressing the induction of inducible nitric oxide synthase and/or proinflammatory cytokines in a cell comprising contacting said cell with an effective amount of at least one induction suppressor and/or inhibitor of inducible nitric oxide synthase. Preferred cells throughout the various embodiments of the invention are lymphocytes, macrophages, endothelial cells, astrocytes, mesangial cells, myocytes, Kuffer cells, epithelial cells, microglia, oligodendrocytes and neurons. Proinflammatory cytokines that are preferred include TNF- α , IL-1 β , IL-2, IL-6, IL-8 and IFN- γ . As used herein certain embodiments "induction" may mean an increase in the overall rate of gene transcription and/or translation. Induction may also mean that the rate of gene message or protein product destruction is decreased, producing a net increase in the amount of a message or translated protein. As used herein certain embodiments, the phrase "inhibition of nitric oxide cytotoxicity" denotes any measurable decrease in the production of NO. Inhibition of nitric oxide cytotoxicity includes inhibition of iNOS activity, production of iNOS protein, production or translation of iNOS mRNA, inhibition of LPS- or cytokine-induced NF-k β activation in a cell. As used herein certain embodiments, "inhibitors" refers to such compounds or agents that produce any measurable decrease in the activity, production, or secretion of a protein or biological compound, or the translation of mRNA, in, or in the case of secretion, from, a cell. Proteins and biological compounds that are specifically contemplated in the invention include iNOS and proinflammatory cytokines. As used herein certain embodiments, a "enhancer" or "stimulator" refers to such compounds or agents that produce any measurable increase in the

activity, production, or secretion of a protein or biological compound, or the translation of mRNA, in, or in the case of secretion, from, a cell. As used herein certain embodiments, an "inducer" refers to such compounds or agents that produce any measurable increase in the content, production, translation, or secretion of a protein or biological compound, or the translation of mRNA, in, or in the case of secretion, from, a cell. As used herein certain
5 embodiments, "a suppressor" refers to an agent or compound that produces any measurable reduction in the induction of a gene. Thus, a "suppressor" is a type of "inhibitor", that acts reduce the net rate of transcription or translation of a target gene.

In preferred aspects of the invention, the induction suppressor and/or inhibitor of
10 inducible nitric oxide synthase and/or proinflammatory cytokines may be selected from the group including, but not limited to, lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), pyrrolidine dithiocarbamate (PDTC), 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives
15 thereof.

In some embodiments, the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be an inhibitor of the Ras/Raf/MAP kinase pathway. In certain embodiments the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be an inhibitor of NF-kB, such as
20 for example an inhibitor of NF-kB activation, and/or a suppressor of its induction. In certain preferred embodiments the inhibitor of NF-kB activation includes, but is not limited to, lovastatin, NaPA, metastatin, 4-phenylbutyrate, FPT inhibitor II, AICAR and salts, analogs, or derivatives thereof. In some embodiments, the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be an inhibitor of
25 mevalonate synthesis. In certain embodiments the inhibitor of mevalonate synthesis may be an inhibitor of the farnasylation of a protein. In certain preferred embodiments the inhibitor of mevalonate synthesis may be an inhibitor of HMG-CoA reductase and/or suppressor of its induction, including but not limited to, lovastatin or AICAR and salts, analogs, or derivatives thereof. In certain preferred embodiments the inhibitor of HMG-CoA reductase is a stimulator
30 of AMP-activated protein kinase, including but not limited to, AICAR and salts, analogs, or derivatives thereof. In certain embodiments, the induction suppressor and/or inhibitor of

inducible nitric oxide synthase and/or proinflammatory cytokines may be a stimulator of AMP-activated protein kinase. In certain other preferred embodiments the inhibitor of of inducible nitric oxide synthase and/or proinflammatory cytokines may be an inhibitor of mevalonate pyrophosphate decarboxylase and/or suppressor of its induction, including but not limited to, phenylacetic acid, 4-phenylbutyrate and salts, analogs, or derivatives thereof. In certain preferred embodiments the inhibitor of mevalonate synthesis may be lovastatin, mevastatin, NaPA, AICAR, 4-phenylbutyrate and salts, analogs, or derivatives thereof. In certain aspects embodiments the inhibitor of of inducible nitric oxide synthase and/or proinflammatory cytokines is an inhibitor of farnesyl pyrophosphate. Preferred inhibitors of farnesyl pyrophosphate include, but are not limited to 4-phenylbutyrate or NaPA.

In other embodiments the suppressor of inducible nitric oxide synthase and/or proinflammatory cytokines is an antioxidant. In preferred embodiments the antioxidant may be, but is not limited to, N-acetyl cysteine, PDTC, and salts, analogs, or derivatives thereof.

In certain other embodiments the inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor is an enhancer of intracellular cAMP, inhibitor of the Ras/Raf/MAP kinase pathway, and/or inhibitor of NF-kB, NF-kB activation and/or suppressor of NF-kB induction. In a preferred embodiment, the inhibitor of the Ras/Raf/MAP kinase pathway includes, but is not limited to, AICAR and salts, analogs, or derivatives thereof. The enhancer of intracellular cAMP may be an inhibitor of cAMP phosphodiesterase and/or suppressor of its induction. In preferred aspects of the invention, the inhibitor of cAMP phosphodiesterase may be, but is not limited to, rolipram and salts, analogs, or derivatives thereof. In certain other aspects of the invention, the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines is cAMP and salts, analogs, or derivatives thereof. Derivatives of cAMP include, but are not limited to, 8-bromo-cAMP or (S)-cAMP. In other aspects of the invention, the enhancer of intracellular cAMP may be, but is not limited to, forskolin, rolipram, 8-bromo-cAMP, theophylline, papaverine, cAMP and salts, analogs, or derivatives thereof. In certain embodiments, the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be a enhancer of protein kinase A. In other aspects of the invention, the enhancer of protein kinase A may include, but is not limited to, forskolin, rolipram, 8-bromo-cAMP, (S)-cAMP, cAMP and salts, analogs, or derivatives thereof. may be, but is not

limited to, forskolin, rolipram, 8-bromo-cAMP, theophylline, papaverine, cAMP and salts, analogs, or derivatives thereof.

In yet another aspect of the invention, the induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be a Ras farnesyl protein transferase inhibitor and/or induction suppressor, an inhibitor of the farnasylation of Ras, and/or an activator of G-proteins. In a preferred embodiment, the Ras farnesyl protein transferase inhibitor and/or induction suppressor includes, but is not limited to, a FPT inhibitor and salts, analogs, or derivatives thereof. In a preferred embodiment, the inhibitor of the farnasylation of Ras, includes, but is not limited to, a FPT inhibitor II and salts, analogs, or derivatives thereof.

In one embodiment of the invention, the inducible nitric oxide synthase and/or proinflammatory cytokines inhibitor and/or induction suppressor is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), PDTC, 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives thereof. In a further embodiment of the invention, combinations of two or more inhibitors and/or induction suppressors are preferred for use in the methods described herein.

A "salt" is understood herein certain embodiments to mean a compound formed by the interaction of an acid and a base, the hydrogen atoms of the acid being replaced by the positive ion of the base. Salts, within the scope of this invention, include both the organic and inorganic types and include, but are not limited to, the salts formed with ammonia, organic amines, alkali metal hydroxides, alkali metal carbonates, alkali metal bicarbonates, alkali metal hydrides, alkali metal alkoxides, alkaline earth metal hydroxides, alkaline earth metal carbonates, alkaline earth metal hydrides and alkaline earth metal alkoxides. Representative examples of bases that form such base salts include ammonia, primary amines such as n-propylamine, n-butylamine, aniline, cyclohexylamine, benzylamine, p-toluidine, ethanolamine and glucamine; secondary amines such as diethylamine, diethanolamine, N-methylglucamine, N-methylaniline, morpholine, pyrrolidine and piperidine; tertiary amines such as triethylamine, triethanolamine, N,N-dimethylaniline, N-ethylpiperidine and N-methylmorpholine; hydroxides such as sodium hydroxide; alkoxides such as sodium ethoxide and potassium methoxide; hydrides such as calcium hydride and sodium hydride; and carbonates such as potassium carbonate and sodium

carbonate. Preferred salts are those of sodium, potassium, ammonium, ethanolamine, diethanolamine and triethanolamine. Particularly preferred are the sodium salts.

As used herein, "derivatives" refers to chemically modified inhibitors or stimulators that still retain the desired effects on property(s) of iNOS or pro inflammatory gene, protein, and/or activity induction or suppression. Derivatives may also retain other desired properties described herein, such as suppressing the accumulation of very long chain fatty acids, defined herein as fatty acids with more than 22 carbon atoms. Such derivatives may have the addition, removal, or substitution of one or more chemical moieties on the parent molecule. Such moieties may include, but are not limited to, an element such as a hydrogen or a halide, or a molecular group such as a methyl group. Such a derivative may be prepared by any method known to those of skill in the art. The properties of such derivatives may be assayed for their desired properties by any means described herein or known to those of skill in the art.

As used herein, "analogs" include structural equivalents or mimetics, described further in the detailed description.

In administering the inducible nitric oxide synthase and/or proinflammatory cytokines inhibitors and/or induction suppressors to a mammal, preferably a human, pig, cats, dogs, rodent, or cattle including but not limited to, sheep, goats and cows, the inhibitor is formulated in a pharmaceutically acceptable vehicle. The induction suppressor and/or inhibitor may be administered to a patient in a dose therapeutic to treat a diseases, conditions and disorders where there is an advantage in inhibiting the nitric oxide synthase enzyme and/or the production of proinflammatory cytokines.

A "patient", as used herein, may be an animal. Preferred animals are mammals, including but not limited to humans, pigs, cats, dogs, rodents, or cattle including but not limited to, sheep, goats and cows. Preferred patients are humans.

The induction suppressors, also known as "suppressing agents", and/or inhibitors of iNOS and/or proinflammatory cytokines, in pure form or in a pharmaceutically acceptable carrier, will find benefit in treating conditions and disorders, described below, where there is an advantage in inhibiting and/or suppression the induction of proinflammatory cytokines and/or the inducible isoform of nitric oxide synthase enzyme. These induction suppressors and/or inhibitors may also be used to treat conditions and disorders created, induced, enhanced and/or aggravated by the contact of a cell with bacterial endotoxin (LPS).

For example, the suppressing agents and/or inhibitors may be used to treat circulatory shock including its various aspects such as vascular and myocardial dysfunction, metabolic failure including the inhibition of mitochondrial enzymes and cytochrome P450-mediated drug metabolism, and multiple organ dysfunction syndrome including adult respiratory distress syndrome. Hypotension and/or circulatory shock may be a result of gram-negative and gram positive sepsis (a.k.a. septic shock), toxic shock, trauma, hemorrhage, burn injury, anaphylaxis, cytokine immunotherapy, liver failure, kidney failure or systemic inflammatory response syndrome. Suppressing agents and/or inhibitors also may be beneficial for patients receiving therapy, including cancer therapy, with cytokines such as TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ , or therapy with cytokine-inducing agents, or as an adjuvant to short term immunosuppression in transplant therapy. In addition, the suppressing agents and/or inhibitors may be useful to inhibit NO synthesis in patients suffering from inflammatory conditions in which an excess of NO contributes to the pathophysiology of the condition, such as adult respiratory distress syndrome (ARDS) and myocarditis, for example.

There is also evidence that an NO synthase enzyme and/or proinflammatory cytokines may be involved in the pathophysiology of autoimmune and/or inflammatory conditions such as arthritis, rheumatoid arthritis and systemic lupus erythematosus (SLE) and in insulin-dependent diabetes, mellitus type 1 diabetes, and therefore, the suppressing agents may prove helpful in treating these conditions.

Furthermore, it is now clear that there are a number of additional inflammatory and noninflammatory diseases and/conditions that are associated with NO overproduction. Examples of such physiological disorders include: inflammatory bowel diseases such as ileitis, ulcerative colitis and Crohn's disease; inflammatory lung disorders such as asthma, bronchitis, oxidant-induced lung injury and chronic obstructive airway disease; inflammatory disorders of the eye including corneal dystrophy, ocular hypertension, trachoma, onchocerciasis, retinitis, uveitis, sympathetic ophthalmitis and endophthalmitis; chronic inflammatory disorders of the gum including periodontitis; chronic inflammatory disorders of the joints including arthritis, septic arthritis and osteoarthritis, tuberculosis, leprosy, glomerulonephritis sarcoid, and nephrosis; disorders of the skin including sclerodermatitis, sunburn, psoriasis and eczema; inflammatory diseases of the central nervous system, including amyotrophic lateral sclerosis, chronic demyelinating diseases such as multiple sclerosis, dementia including AIDS-related

neurodegeneration and Alzheimer's disease, encephalomyelitis and viral or autoimmune encephalitis; autoimmune diseases including immune-complex vasculitis, systemic lupus and erythematosus; and disease of the heart including ischemic heart disease, heart failure and cardiomyopathy. Additional disease that may benefit from the use of suppressing agents include

5 adrenal insufficiency; hypercholesterolemia; atherosclerosis; bone disease associated with increased bone resorption, *e.g.*, osteoporosis, pre-eclampsia, eclampsia, uremic complications; chronic liver failure, noninflammatory diseases of the central nervous system (CNS) including stroke and cerebral ischemia; and other disorders associated with inflammation and undersirable production of nitric oxide and/or proinflammatory cytokines such as cystic fibrosis,

10 tuberculosis, cachexia, ischemia/reperfusion, hemodialysis related conditions, glomerulonephritis, restenosis, inflammatory sequelae of viral infections, hypoxia, hyperbaric oxygen convulsions and toxicity, dementia, Sydenham's chorea, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, epilepsy, Korsakoff's disease, imbecility related to cerebral vessel disorder, NO mediated cerebral trauma

15 and related sequelae, ischemic brain edema (stroke), pain, migraine, emesis, immune complex disease, as immunosuppressive agents, acute allograft rejection, infections caused by invasive microorganisms which produce NO and for preventing or reversing tolerance to opiates and diazepam, aging, and various forms of cancer. All these nitric oxide and/or proinflammatory cytokine and/or endotoxin induced, mediated, enhanced, and/or aggravated diseases and

20 disorders are contemplated as being treatable in a cell by contacting the cell with at least one suppressing agent and/or inhibitor of iNOS and/or proinflammatory cytokines. A patient with may also be treated by administering at least one suppressing agent and/or inhibitor of iNOS and/or proinflammatory cytokines. When administered to a patient, the at least one suppressing agent and/or inhibitor is formulated in a pharmaceutically acceptable vehicle.

25 In another aspect the present invention provides a method of identifying, or screening for, a candidate inducible nitric oxide synthase and/or proinflammatory cytokines inhibitor and/or induction suppressor, comprising preparing a cell capable of producing inducible nitric oxide synthase and/or proinflammatory cytokines activity and testing the candidate inhibitor and/or induction suppressor for the ability to inhibit the inducible nitric oxide synthase and/or

30 proinflammatory cytokines activity, wherein the inhibition is indicative of a candidate inducible nitric oxide synthase and/or proinflammatory cytokines inhibitor and/or induction suppressor.

These candidate inhibitor and/or induction suppressor are known herein as "candidate substances". A further aspect of this method is to identify an iNOS specific inhibitor and/or induction suppressor that does not inhibit or suppress one or more proinflammatory cytokines. Another aspect of this invention is to identify an inhibitor and/or induction suppressor that does not inhibit or suppress iNOS, but does inhibit or suppress one or more proinflammatory cytokines.

This method of identifying a candidate inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor comprising the steps of a) obtaining a cell comprising at least the capability of producing inducible nitric oxide synthase and/or proinflammatory cytokines activity; b) obtaining a candidate inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor; c) contacting the cell with the candidate inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor under conditions normally inducing, enhancing, and/or stimulating iNOS and/or proinflammatory cytokines; and d) determining the ability of the candidate inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor to inhibit the formation of nitric oxide in the presence of inducible nitric oxide synthase, wherein the inhibition of the formation of nitric oxide in the presence of inducible nitric oxide synthase is indicative of a candidate inducible nitric oxide synthase induction suppressor and/or inhibitor. In an aspect of the invention, decreased content or production of at least one proinflammatory cytokine by a cell is indicative of a candidate proinflammatory cytokine induction suppressor. In another aspect of the invention, decreased bioactivity of at least one proinflammatory cytokine is indicative of a candidate proinflammatory cytokine inhibitor and/or induction suppressor. In further aspects of this method, an induction suppressor and/or inhibitor is further identified by detecting the amount of iNOS and/or proinflammatory cytokine mRNA message and/or protein content and/or biological activity. In additional aspects of the invention, an induction suppressor and/or inhibitor is further identified by comparing the amount of iNOS and/or proinflammatory cytokine mRNA message and/or protein content and/or biological activity to another cell under conditions normally inducing, enhancing, and/or stimulating iNOS and/or proinflammatory cytokines in the absense of the candidate inhibitor and/or induction suppressor. The preferred conditions inducing, enhancing, and/or stimulating iNOS and/or proinflammatory cytokines is

contacting a cell with endotoxin and/or at least one cytokine and/or at least one inducer or stimulator of at least one proinflammatory cytokine. Preferred cytokines are proinflammatory cytokines.

In preferred embodiments, a candidate induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines is selected from agents that have certain traits or modes of action common to those of the suppressors and/or inhibitors identified herein. Preferred candidate substances would either inhibit the Ras/Raf/MAP kinase pathway, inhibit and/or suppress the induction and/or activation of NF-kB, inhibit mevalonate synthesis, be an enhancer of protein kinase A, and/or inhibit the farnasylation of proteins, including but not limited to Ras. In certain embodiments the inhibitor of mevalonate synthesis may be an inhibitor of HMG-CoA reductase or suppressor of its induction. In certain aspects the inhibitor of HMG-CoA reductase is a stimulator of AMP-activated protein kinase. In certain other embodiments the inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines may be an inhibitor of mevalonate pyrophosphate decarboxylase or suppressor of its induction. In other embodiments the candidate substance is an antioxidant. In other embodiments the candidate substance is an enhancer of intracellular cAMP. The enhancer of intracellular cAMP may be an inhibitor of cAMP phosphodiesterase and/or suppressor of its induction. In other embodiments the candidate substance is a farnesyl protein transferase inhibitor and/or induction suppressor.

Proinflammatory cytokine and/or iNOS RNA message, protein content, or activity can be detected by any method described herein or known to those of skill in the art (see for example, Sambrook *et al.*, 1989), and include but are not limited to Northern analysis of iNOS and/or inflammatory cytokine message, PCR™ amplification of target message, immunodetection techniques including Western analysis of iNOS and/or proinflammatory cytokine content or production, and chemical or biological activity assays for iNOS or cytokine activity.

Candidate inhibitors and/or induction suppressors identified by the method of the invention are preferably purified. When administered to a mammal, the purified candidate inducible nitric oxide synthase inhibitor and/or induction suppressor is formulated in a pharmaceutically acceptable vehicle.

In another preferred embodiment, the invention provides a method of inhibiting nitric oxide cytotoxicity comprising contacting a cell capable of producing nitric oxide with a biologically effective amount of at least one inducible nitric oxide synthase induction suppressor and/or inhibitor identified by the screening assay of the invention. In preferred
5 embodiments, the cell is in a patient.

In another preferred embodiment, the invention provides a method of inhibiting proinflammatory cytokine or endotoxin treated, induced or aggravated conditions and disorders, where there is an advantage in inhibiting and/or suppression the induction of proinflammatory cytokines. In certain embodiments, the method comprises contacting a cell with a biologically
10 effective amount of at least one induction suppressor and/or inhibitor of: at least one proinflammatory cytokine and/or iNOS. In certain aspects of the invention, the at least one induction suppressor and/or inhibitor is identified by the screening assay of the invention. In preferred embodiments, the cell is in a patient.

The invention also provides a method of suppressing the accumulation of very long
15 chain fatty acids in a cell, by contacting the cell with a biologically effective amount of at least induction suppressor and/or inhibitor of: inducible nitric oxide synthase and/or at least one proinflammatory cytokine. In certain aspects of the invention, the at least one induction suppressor and/or inhibitor is identified by the screening assay of the invention. In preferred
20 embodiments, the cell is in a patient. Such methods have use in inflammatory conditions including, but not limited to, demyelinating diseases or neural trauma, and particularly in treating patients with X-ALD. In certain aspects of the invention, lignoceric acid β -oxidation is stimulated. In other aspects of the invention, the ratios of $C_{26:0}/C_{22:0}$ or $C_{24:0}/C_{22:0}$ fatty acids are lowered.

The invention provides a method of treating a nitric oxide and/or cytokine mediated
25 disorder in a cell, by contacting the cell with a biologically effective amount of at least one induction suppressor and/or inhibitor of: inducible nitric oxide synthase and/or at least one proinflammatory cytokine. In certain aspects of the invention, the at least one induction suppressor and/or inhibitor is identified by the screening assay of the invention. In preferred
30 embodiments, the cell is in a patient. In preferred aspects, the disorder is X-ALD, multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, lupus, septic shock, stroke, ischemia/reperfusion, rheumatoid arthritis, osteoarthritis or aging. In other preferred aspects,

the nitric oxide or cytokine mediated disorder is myelinolytic inflammation, a demyelinating condition or an inflammatory demyelinating disease, or a neuroinflammatory disease. The inflammatory disease is preferably X-ALD, multiple sclerosis, Landry-Guillain-Barre-Strohl syndrome, Alzheimer's disease and/or aging.

5 In another preferred embodiment, the invention provides a method of treating septic shock comprising contacting a cell capable of producing excess nitric oxide and/or at least one proinflammatory cytokine under conditions of septic shock with a biologically effective amount of an inducible nitric oxide synthase and/or proinflammatory cytokine induction suppressor and/or inhibitor. In certain aspects of the invention, the induction suppressor and/or inhibitor is
10 identified by the screening assay of the invention. In preferred aspects of the invention, the cell is in a patient. Methods of treating septic shock with inhibitors of nitric oxide synthase activity are described in U.S. Patents Nos. 5,028,627 and 5,296,466, each incorporated herein by reference in entirety.

The present invention is further directed to methods for inducing or suppressing
15 apoptosis in the cells and/or tissues of individuals suffering from degenerative disorders characterized by inappropriate cell proliferation or inappropriate cell death, or in some cases, both. The method comprises contacting a cell capable of producing excess nitric oxide under conditions of degenerative disorders with a biologically effective amount of an inducible nitric oxide synthase and/or proinflammatory cytokines induction suppressor and/or inhibitor. In
20 preferred aspects of the invention, the cell is in a patient. In certain aspects of the invention, the cytokines induction suppressor and/or inhibitor identified by the screening assay of the invention. Inappropriate cell proliferation will include the statistically significant increase in cell number as compared to the proliferation of that particular cell type in the normal population. Also included are disorders whereby a cell is present and/or persists in an
25 inappropriate location, *e.g.*, the presence of fibroblasts in lung tissue after acute lung injury, and cancer cells which exhibit the properties of invasion and metastasis and are highly anaplastic. Such cells include but are not limited to, cancer cells including, for example, tumor cells. Inappropriate cell death will include a statistically significant decrease in cell number as compared to the presence of that particular cell type in the normal population. Such
30 underrepresentation may be due to a particular degenerative disorder, including, for example, viral infections such as AIDS (HIV), which results in the inappropriate death of T-cells, and

autoimmune diseases which are characterized by inappropriate cell death. Autoimmune diseases are disorders caused by an immune response directed against self antigens. Such diseases are characterized by the presence of circulating autoantibodies or cell-mediated immunity against autoantigens in conjunction with inflammatory lesions caused by immunologically competent cells or immune complexes in tissues containing the autoantigens. Such diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis. Standard reference works setting forth the general principles of immunology include Stites and Terr, 1991 and Abbas *et al.*, 1991.

The invention particularly relates to the use of at least one iNOS and/or pro-inflammatory cytokine induction suppressor and/or inhibitors, preferably reductants such as NAC or other thiol compounds to reduce NO-mediated cytotoxicity as well as ceramide-mediated apoptosis in neuroinflammatory diseases and degenerative disorders. Suppressing agents in this class would be particularly preferred in treating diseases characterized by excessive or inappropriate cell death, including, for example, neurodegenerative diseases and injury resulting from ischemia. Degenerative disorders characterized by inappropriate cell proliferation include, for example, inflammatory conditions, cancer, including lymphomas, such as prostate hyperplasia, genotypic tumors, etc. Degenerative disorders characterized by inappropriate cell death include, for example, autoimmune diseases, acquired immunodeficiency disease (AIDS), cell death due to radiation therapy or chemotherapy, neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Landry-Guillain-Barre-Strohl syndrome, multiple sclerosis, etc. In certain aspects of the invention, the at least one induction suppressor and/or inhibitor is identified by the screening assay of the invention.

The invention further provides a method for enhancing the production of an inducible nitric oxide synthetase or a proinflammatory cytokine in a cell comprising providing a biologically effective amount of a inducible nitric oxide synthetase and/or proinflammatory cytokine stimulator. In certain aspects of the invention, the at least one induction stimulator and/or enhancer is identified by the screening assay of the invention. A stimulator in this aspect of the invention is preferably an induction stimulator. Preferred stimulators include a PKA inhibitor or enhancer of intracellular cAMP. PKA inhibitors may include, but are not limited to, H-89, myristoylated PKI, (R)-cAMP and salts, analogs, or derivatives thereof. The

enhancers of intracellular cAMP may also be selected from the group comprising forskolin, 8-bromo-cAMP and rolipram. In other preferred aspects of the invention, the enhancer of intracellular cAMP is an inhibitor of cAMP phosphodiesterase. A preferred inhibitor of cAMP phosphodiesterase is rolipram. In other aspects of this method, a biologically effective amount of LPS and/or one or more proinflammatory cytokine is administered to stimulate iNOS and/or proinflammatory cytokines' induction or activity. Preferred proinflammatory cytokine that are administered include TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ .

Following long-standing patent law convention, the words "a" and "an," as used in this specification, including the claims, denotes "one or more." Specifically, the use of "comprising," "having," or other open language in claims that claim a combination or method employing "an object," denotes that "one or more of the object" may be employed in the claimed method or combination.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Forskolin inhibits LPS-induced NO production and iNOS activity in a dose-dependent manner in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 received different concentrations of forskolin 15 min before the addition of 1.0 μ g/ml LPS. The production of nitrite in supernatants (○) and activities of iNOS in cell homogenates (●) were measured after 24 h of incubation as described in Example 6. PKA activity was measured in cell homogenates (□) after 1 h of incubation. Nitrite production in supernatants (32.3 ± 3.6 nmol/mg/24 h), and iNOS activity in homogenates (48.7 ± 3.9 pmol/min/mg) found in cells stimulated with only LPS are considered as 100%. However, PKA activity found in extracts from cells stimulated with an optimal concentration of forskolin (74.4 ± 9.4 pmol/min/mg) is considered as 100%. Values are mean of duplicate samples.

FIG. 2. Activation of PKA correlates with the stimulation of β -oxidation and inhibition of fatty acid chain elongation in cultured skin fibroblasts of X-ALD. Cells were treated for 72 h serum-containing DMEM with the listed reagents; β -oxidation of lignoceric

acid (FIG. 2A), fatty acid chain elongation (FIG. 2B) and PKA (FIG. 2C) activities were measured as described in Example 4. Media was replaced after every 24 h with the addition of fresh reagents. Concentrations of reagents were: forskolin, 4 μ M; 8-Br-cAMP, 50 μ M; rolipram, 10 μ M; H-89, 1 μ M; myristoylated PKI, 0.2 μ M. Data are mean \pm S.D. of three different experiments.

FIG. 3. Time-dependent effect of forskolin on the ratios of VLCFA ($C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$) and β -oxidation of lignoceric acid in cultured skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM with 4 μ M forskolin for different days, and the ratios of $C_{26:0}/C_{22:0}$ (FIG. 3A) and $C_{24:0}/C_{22:0}$ (FIG. 3B), and β -oxidation of lignoceric acid (FIG. 3C) were measured as described in Example 4 (O, experiment 1; O, experiment 2).

FIG. 4. Modulators of PKA modulate the induction of TNF- α and IL-1 β in rat primary astrocytes. Cells preincubated with the listed reagents for 15 min in serum-free condition was stimulated with 1.0 μ g/ml of LPS. After 24 h of incubation, concentrations of TNF- α (FIG. 4A) and IL-1 β (FIG. 4B) were measured in supernatants as described in the methods section. After 1 h of incubation, activity of PKA (FIG. 4C) was measured in cell extracts as described in Example 4. TNF- α and IL-1 β are expressed as ng/24 h/mg protein. Data are expressed as the mean \pm S.D. of three different experiments. Concentrations of reagents were: forskolin, 10 μ M; 8-Br-cAMP, 100 μ M; rolipram, 20 μ M; H-89, 2 μ M; myristoylated PKI, 0.4 μ M. Data are mean \pm S.D. of three different experiments.

FIG. 5. Effect of okadaic acid on iNOS promoter-derived CAT activity in rat primary astrocytes and macrophages. Astrocytes (FIG. 5A) macrophages (FIG. 5B) were transfected with the construct containing the iNOS promoter fused to the CAT gene using lipofectamine. Twenty four hour after transfection, cells received okadaic acid with or without 1.0 μ g/ml of LPS and after 14 h of stimulation, CAT activity was measured. Data are mean \pm S.D. of three different experiments.

FIG. 6. Inhibition of TNF- α -induced degradation of sphingomyelin to ceramide by NAC and PDTC in rat primary astrocytes. Cells preincubated with either 10 mM NAC or 100 μ M PDTC for 1 h in serum-free DMEM/F-12 received TNF- α (50 ng/ml). At different time intervals, cells were washed with HBSS and scrapped off Lipids were extracted, and levels of ceramide (FIG. 6A) and sphingomyelin (FIG. 6B) were measured as described in Example 7.

Ceramide levels are expressed as -fold change over the level at 0 min. Results are mean \pm S.D. of three different studies.

FIG. 7. NAC and PDTC inhibit IL-1 β -mediated degradation of sphingomyelin to ceramide in rat primary astrocytes. Cells preincubated with either 10 mM NAC or 100 μ M PDTC for 1 h in serum-free DMEM/F-12 received IL-1 β (50 ng/ml). At different time intervals, cells were washed with HBSS and scrapped off Lipids were extracted, and levels of ceramide (FIG. 7A) and sphingomyelin (FIG. 7B) were measured as described in Example 7. Ceramide levels are expressed as -fold change over the level at 0 min. Results are mean \pm S.D. of three different studies.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The invention discloses the novel uses of compounds which inhibit the induction of iNOS and/or proinflammatory cytokines and the production of NO and/or proinflammatory cytokines by cells, including lymphocytes, macrophages, endothelial cells, astrocytes and microglia in response to inflammatory cytokines for the therapeutic treatment of disease affecting the vascular and nervous systems. The therapeutic uses described herein utilizing these compounds provide protection against NO toxicity to including lymphocytes, macrophages, endothelial cells, astrocytes microglia, oligodendrocytes and neurons in neuroinflammatory disease, stroke, ischemia-reperfusion and tissue injury and HIV-mediated NO neurotoxicity for which there is no effective treatment presently available.

The present disclosure further describes the discovery of a novel role of the mevalonate pathway in controlling the expression of iNOS and different cytokines in lymphocytes, macrophages, endothelial cells, astrocytes and microglia. This discovery provides the basis for novel screening assays of previously unknown inhibitors of iNOS and the production of NO. An understanding of the cellular mechanisms involved in the induction of iNOS and cytokines allows identification of novel targets for the therapeutic intervention of NO-mediated, proinflammatory cytokine and/or endotoxin-mediated pathophysiology in inflammatory diseases.

The inventor demonstrates herein that LPS- and cytokine-induced production of NO can be blocked by antioxidants. Therefore maintenance of the thiol/oxidant balance appears to be crucial for protection against proinflammatory cytokine production and, at least, in NO

cytotoxicity. The inventor has discovered that the use of reductants, such as N-acetyl cysteine (NAC) or other thiol compounds, is beneficial in restoring cellular redox and in inhibiting the production of proinflammatory cytokines and in reducing cytotoxic levels of NO. N-acetyl cysteine blocks the induction of TNF- α and iNOS and is a nontoxic drug that enters the cell readily and serves both as a scavenger of reactive oxygen species and a precursor of glutathione, the major intracellular thiol (Smilkstein *et al.*, 1988; Aruoma *et al.*, 1989). Therefore, the use of reductants such as NAC or other thiol compounds, may be beneficial in restoring cellular redox and in inhibition of production of proinflammatory cytokines and in reducing cytotoxic levels of NO.

The inventor investigated the cellular regulation of the induction of iNOS and cytokines by lovastatin and NaPA in rat primary lymphocytes, macrophages, endothelial cells, astrocytes and microglia. This investigation disclosed the first evidence that the induction of inducible nitric oxide synthase (iNOS) and cytokine (for example TNF- α , IL-1 β and IL-6) gene expression are uniquely sensitive to the drugs lovastatin and the sodium salt of phenylacetic acid (NaPA) in astrocytes, glial cells and macrophages. The reversal of lovastatin-mediated inhibition of iNOS induction by mevalonate and FPP, and reversal of the inhibitory effect of NaPA by FPP, and inhibition of Ras farnesyl protein transferase by an inhibitor (FPT inhibitor II) demonstrated that the farnesylation reaction is a key step in the regulation of LPS-mediated induction of iNOS and production of NO and cytokines.

The inventor have discovered that lovastatin and NaPA, alone or in combination represent therapeutic agents directed against cytokine- and nitric oxide-mediated brain disorders, particularly in stroke, trauma, Alzheimer's Disease and in demyelinating conditions such as multiple sclerosis and X-adrenoleukodystrophy (X-ALD).

The inventor's results demonstrate that the inhibition iNOS expression by lovastatin, NaPA and FPT inhibitor II may be due to the inhibition of NF- κ B activation. Previous studies of Law *et al.* (1992) demonstrating the inhibition of NF- κ B activation by mevinolin and 5'-methylthioadenosine indicated a role of protein farnesylation and carboxyl methylation reactions in the activation of NF- κ B. The Ras protooncogene proteins function by binding to cytoplasmic surface of plasma membrane. Since mevalonate availability regulates the post-translational isoprenylation of many intracellular signaling proteins including Ras p21 (Goldstein *et al.*, 1990), the observed inhibition of NF- κ B activation and induction of iNOS by

lovastatin and NaPA appears to be due to decreased, or a lack of, isoprenylation of Ras that in turn leads to the lack of or abnormal signal transmission from receptor tyrosine kinase to Ras/Raf/MAP kinase cascade, activation of NF- κ B and induction of iNOS.

The inventor has also investigated the effect of other antioxidants on the induction of NO by LPS and/or cytokine-stimulated macrophages, C6 glioma cell lymphocytes, endothelial cells, astrocytes and microglia. These results clearly show that antioxidants (N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC)) inhibit the LPS- and cytokine-induced production of NO, iNOS activity, production of iNOS protein and iNOS mRNA indicating a role of reactive oxygen species (*e.g.*, H₂O₂, O₂ and OH) in iNOS induction. Superoxide (O₂⁻) and hydroxyl radical (OH) are reported to be involved in the production of NO in brain cerebellum (Mittal, 1993) where the hydroxyl radical was indicated to hydroxylate L-arginine during its conversion to citrulline and NO (Mittal, 1993). The inventor has discovered through the inhibition of iNOS activity and induction of iNOS protein and mRNA in LPS- and cytokine-activated macrophages by NAC that reactive oxygen species (ROS) modulate the intracellular signal pathways for the induction of iNOS biogenesis.

Several lines of evidence disclosed herein clearly support the conclusion that inhibitors of HMG-CoA reductase (for example, lovastatin or mevastatin) and mevalonate pyrophosphate decarboxylase (NaPA) have an inhibitory effect on the induction of inflammatory mediators (iNOS, TNF- α , IL-1 β and IL-6) in rat astrocytes, microglia and macrophages demonstrating the involvement of mevalonate metabolite(s), farnesyl pyrophosphate, in the induction of inflammatory mediators. This conclusion was based on the following observations. First, the LPS-induced expression of iNOS, TNF- α , IL-1 β and IL-6, and activation of NF- κ B was inhibited by lovastatin and NaPA. Second, inhibitory effects of lovastatin and NaPA on LPS-mediated induction of iNOS and cytokines was not reversed by cholesterol and ubiquinone, end products of the mevalonate pathway, indicating that this inhibitory effect of lovastatin was not due to depletion of end products of mevalonate pathway. Third, the reversal of inhibitory effects of lovastatin by mevalonate and FPP and the reversal of inhibitory effects of NaPA by FPP, but not by mevalonate, indicates a role of farnesylation in LPS-mediated induction of iNOS. Fourth, the inhibition of LPS-induced activation of NF- κ B and induction of iNOS by FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, demonstrates that farnesylation of Ras is required for signal transduction in the LPS-induced expression of iNOS.

Since the iNOS, TNF- α , IL-1 β and IL-6 have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases (Mitrovic *et al.*, 1994; Bo *et al.*, 1994; Merrill *et al.*, 1993), these results provide an important mechanism whereby inhibitors of HMG-CoA reductase and mevalonate pyrophosphate decarboxylase can ameliorate neural injury.

Therapy For X-Adreno Leukodystrophy

Since X-ALD is a metabolic disorder of the very long chain fatty acids (VLCFA) that eventually leads to an inflammatory bilateral demyelination with marked activation of microglia and astrocytes and accumulation of proinflammatory cytokines (TNF- α and IL-1 β) and extracellular matrix proteins (Powers *et al.*, 1992; McGuinness *et al.*, 1995), the inventor developed a therapy that should normalize the VLCFA and inhibit the induction of proinflammatory cytokines by astrocytes and microglia. Example 4 described herein demonstrates that the compounds that increase the intracellular levels of cAMP and the activity of protein kinase A (PKA) normalize the levels of VLCFA possibly by increasing the peroxisomal activity for β -oxidation of VLCFA. Moreover, the same compounds also inhibit the induction of TNF- α and IL-1 β in lipopolysaccharide (LPS) stimulated astrocytes and microglia. These observations demonstrate the therapeutic potential of compounds that increase the activity of PKA in correction of the metabolic defect and inhibition of the neuroinflammatory disease process in X-ALD.

The inventor provides evidence that in X-ALD cultured skin fibroblasts, up regulation of PKA activity increased the β -oxidation of lignoceric acid, decreased the chain elongation of fatty acids and lowered cellular content of VLCFA to the normal level, despite the status (mutation or deletion) of the ALD gene. The detailed mechanism leading to the normalization of VLCFA in X-ALD is not known at the present, but is likely to involve cAMP-dependent protein kinase A. This conclusion is based on the following observations. First, cAMP analogs and rolipram, an inhibitor of cAMP phosphodiesterase, stimulated transport and β -oxidation of lignoceric acid and decreased the chain elongation of fatty acids in X-ALD as well as control skin fibroblasts whereas H-89 and myristoylated PKI, specific inhibitors of PKA, inhibited transport and β -oxidation of lignoceric acid, stimulated chain elongation of fatty acids and blocked the observed effects in normalization of VLCFA by cAMP analogs. Second, a

long-term treatment of fibroblasts of X-ALD with cAMP analogs and rolipram although had no effect on protein and mRNA for X-ALD gene but lowered the accumulation of VLCFA to the control level that is also blocked by inhibitors of PKA. These results clearly indicate that increasing cAMP level in fibroblasts of X-ALD normalizes the VLCFA pathogen by a mechanism that is dependent on the activity of PKA but independent of the involvement of the ALD gene product.

Previous studies (Singh *et al.*, 1984; Hashmi *et al.*, 1986; Lageweg *et al.*, 1991; Lazo *et al.*, 1988; Lazo *et al.*, 1989) have shown that VLCFA (lignoceric and cerotic acids) are preferentially β -oxidized in peroxisomes. The increased transport of lignoceric acid into cAMP-treated cells indicates that the observed increase in β -oxidation of lignoceric acid is due to higher availability of lignoceric acid in these cells. However, the increase in β -oxidation of lignoceric acid in cell-free extracts or permeabilized X-ALD cells, or cell homogenates demonstrate that normalization of VLCFA is due to increased activity of fatty acid β -oxidation system. In the cell, fatty acids are β -oxidized in mitochondria and peroxisomes (Singh, 1997). The lack of effect of etomoxir, an inhibitor of mitochondrial carnitine palmitoyl transferase-I (Mannaerts *et al.*, 1979), on the cAMP-stimulated oxidation indicates that the higher lignoceric acid oxidation activity observed in cAMP-stimulated cells was due to increase in the activity of peroxisomal β -oxidation system. These observations provide the first evidence that peroxisomal β -oxidation of fatty acids is regulated by intracellular second messenger (cAMP).

The pathogenetic mechanism of X-ALD is poorly understood. The constant "hallmark" of X-ALD is an excessive accumulation of VLCFA with subsequent involvement of CNS with induction of proinflammatory cytokines (TNF- α and IL-1 β) and extracellular matrix proteins by reactive astrocytes and microglia and demyelination/inflammatory dysmyelination and loss of oligodendrocytes (Powers *et al.*, 1992; McGuinness *et al.*, 1995; Powers, 1995). The documentation of immunoreactive TNF- α and IL-1 β in astrocytes and microglia of X-ALD brain indicated the involvement of these cytokines in immunopathology of X-ALD and aligned X-ALD with multiple sclerosis (MS), the most common immune-mediated demyelinating disease of the CNS in man. However, apart from traditionally higher expression of cytokines by microglia than in astrocytes of MS and other neurodegenerative disorders, the expression of TNF- α and IL-1 β is more prominent in astrocytes than microglia of X-ALD brain (Powers *et al.*, 1992).

At present it is not known how the inherited metabolic abnormality of accumulation of VLCFA subsequently triggers a neuroinflammatory response in X-ALD brain. Since the metabolic defect appears prior to the detection of neuroinflammatory disease, the assumption is that these VLCFA, by themselves or as a constituent of complex lipid, act as a trigger for the inflammatory response that in turn becomes the basis for the observed demyelination and loss of oligodendrocytes in X-ALD. The data presented here indicate that cAMP may also inhibit the induction of proinflammatory cytokines in reactive astrocytes and microglia. The treatments of rat brain primary astrocytes or microglia with forskolin or rolipram inhibit the LPS-induced induction of TNF- α and IL-1 β .

Previously it has been shown that cAMP derivatives and rolipram inhibit the cytokine-induced expression of inducible nitric oxide synthase and production of NO in astrocytes. The inventor's studies indicate that proinflammatory cytokines down regulate the peroxisomal function in the metabolism of VLCFA thereby aggravating the inherited metabolic abnormality by accumulating 4-times higher VLCFA and around the plaque than in normal looking X-ALD brain and these alterations by proinflammatory cytokines are mediated by NO toxicity (Khan *et al.*, 1997). The inhibition of induction of cytokines as well as induction of iNOS by compounds that increase the activity of PKA (*e.g.*, cAMP and rolipram) in astrocytes and microglia indicate that these compounds should be beneficial in terms of blocking the induction of proinflammatory cytokines in X-ALD

These results provide the basis of a therapy to normalize the metabolic abnormality and block the neuroinflammatory process by inhibiting the induction of proinflammatory cytokines. The studies described in Example 4 clearly demonstrate that the compounds (*e.g.* forskolin, 8-Br-cAMP, rolipram) that increase cAMP and activate PKA meet both of these conditions. Moreover, recent reports showing the prevention of progression of autoimmune encephalomyelitis in mice (Sommer *et al.*, 1995) as well as in marmoset by rolipram indicate that rolipram does cross the blood brain barrier and inhibit the cytokine-induced neuropathologies in these animal models.

The studies described in Example 5 demonstrate that lovastatin and sodium phenylacetate (NaPA), inhibitors of mevalonate pathway, normalize the levels of VLCFA in skin fibroblasts of X-ALD by increasing the peroxisomal activity for β -oxidation of VLCFA. In light of the fact that these compounds also inhibit the induction of proinflammatory cytokines

and nitric oxide synthase in astrocytes and microglia, the inventor deduced that these drugs may have therapeutic potential in correction of the metabolic defect and inhibition of the neuroinflammatory disease process in X-ALD.

The inventor found that PD 98059, an inhibitor of MAP kinase (MEK), the kinase responsible for the activation of MAP kinase, inhibits the LPS-induced activation of NF-kB and the induction of iNOS in astrocytes indicating the possible involvement of the MAP kinase pathway in the induction of iNOS. MAP kinases exhibit dual-specificity, regulating both serine (Ser)/threonine (Thr) phosphorylation and Tyr autophosphorylation (Blenis, 1993; Rossomando *et al.*, 1994; Her *et al.*, 1993). In addition, MAP kinases themselves require concurrent Thr and Tyr phosphorylation for activation, and are, in turn, substrates for MEK (Blenis, 1993; Rossomando *et al.*, 1994; Her *et al.*, 1993). MEK is also a dual specificity kinase whose activation requires Ser/Thr phosphorylation (Blenis, 1993; Rossomando *et al.*, 1994; Her *et al.*, 1993). The inventor deduced from these observations that cellular regulation of this signaling pathway may utilize Ser/Thr phosphatases to modulate the phosphorylation state of critical phosphoproteins.

Since phosphoprotein phosphatases (PP) 1 and PP 2A are the two most abundant Ser/Thr phosphatases in the cell, the study presented in Example 6 was undertaken to investigate the cellular regulation of the induction of iNOS by PP 1 and PP 2A in rat primary astrocytes and macrophages. The results clearly demonstrate that calyculin A, microcystin, cantharidin and okadaic acid, inhibitors of PP 1 and PP 2A, stimulate the LPS- and cytokine-mediated expression of iNOS and production of NO in astrocytes and C₆ glial cells while the same inhibitors inhibit the LPS- and cytokine-mediated expression of iNOS and production of NO in macrophages and RAW 264.7 cells. Consistent with this observation, okadaic acid stimulates the iNOS promoter-derived chloramphenicol acetyl transferase (CAT) activity in LPS-treated astrocytes but inhibits the iNOS promoter-derived CAT activity in LPS-treated macrophages. This differential regulation of the induction of iNOS in astrocytes and macrophages by inhibitors of PP 1/2A indicates that, although PP 1/2A functions as a physiological inhibitor of the induction of iNOS in astrocytes, the induction of iNOS in macrophages requires the involvement of PP 1/2A. However, in spite of this differential regulation of the induction of iNOS in astrocytes and macrophages, inhibitors of PP 1/2A

stimulate the activation of NF-kB and the production of TNF- α in both astrocytes and macrophages.

Transient modulation of protein phosphorylation and dephosphorylation is a major mechanism of intracellular signal transduction pathways triggered by different cytokines.

5 Therefore, the inventor hypothesized that inhibition of protein phosphatase 1 and 2A (PP 1 and 2A) activities will influence cytokine induced signal transduction pathways for the induction of iNOS. The signaling events in cytokine-mediated induction of iNOS in astrocytes and macrophages are not well understood. An understanding of the cellular mechanisms involved in the induction of iNOS should identify novel targets for therapeutic intervention in NO-mediated
10 neuroinflammatory diseases. Several lines of evidence presented in Example 6 support the conclusion that inhibition of PP 1/2A activity differentially modulates the LPS- and cytokine-induced expression of iNOS and production of NO in rat primary astrocytes and macrophages. The conclusion is based on the following observations. First, treatment of astrocytes and macrophages with LPS and/or cytokines induced the expression of iNOS and
15 production of NO, and inhibitors of PP 2B (cypermethrin, deltamethrin and fenvalerate) had no effects on the LPS- and cytokine-mediated induction of iNOS and production of NO. Second, compounds (calyculin A, microcystin, okadaic acid and cantharidin) that inhibit PP 1/2A stimulated the LPS- and cytokine-mediated production of NO as well as expression of iNOS protein and mRNA in astrocytes and C₆ glial cells. However, in contrast, these inhibitors
20 inhibited the LPS- and cytokine-mediated production of NO and expression of iNOS in rat resident macrophages and RAW 264.7 cells. Third, the inhibitors of PP 1/2A stimulated iNOS promoter-derived chloramphenicol acetyl transferase (CAT) activity in LPS-treated astrocytes but inhibited iNOS promoter-derived CAT activity in LPS-treated macrophages. These results indicate that the signaling events required for the induction of iNOS in astrocytes differ from
25 those required for the induction of iNOS in macrophages.

Cytokines (TNF- α , IL-1 β or IFN- γ) and LPS bind to their respective receptors and induce iNOS expression via activation of NF-kB (Xie *et al.*, 1994; Kwon *et al.*, 1995). The nuclear expression and biological function of the NF-kB transcription factor are tightly regulated through its cytoplasmic retention by the ankyrin-rich inhibitor I κ B α (Beg *et al.*,
30 1992). Activation of NF-kB by various cellular stimuli involves the proteolytic degradation of I κ B α and the concomitant nuclear translocation of the liberated NF-kB heterodimer. Although

the biochemical mechanism underlying the degradation of I κ B α remains unclear, it appears that degradation of I κ B α induced by various mitogens and cytokines occurs in association with the transient phosphorylation of I κ B α on serines 32 and 36. Further the inventor has found that the 90 kDa ribosomal S6 kinase (a downstream candidate of the well characterized
5 Ras-Raf-MEK-MAP kinase pathway), but not p70 S6 kinase or MAP kinase, phosphorylates the N-terminal regulatory domain of I κ B α on serine 32. However, *in vivo*, only phorbol 12-myristate 13-acetate produced rapid activation of p90 RSK, other potent NF- κ B inducers including TNF- α and the Tax transactivator of human T-cell lymphotropic virus, type I, failed to activate p90 RSK indicating that more than a single I κ B α kinase exists within the cell and
10 that these I κ B α kinases are differentially activated by different NF- κ B inducers. By phosphorylation, I κ B α which is still bound to NF- κ B has apparently turned into a high affinity substrate for an ubiquitin-conjugating enzyme. Following this phosphorylation-controlled ubiquitination, I κ B α is rapidly and completely degraded by the 20 S or 26 S proteasome.

Okadaic acid and other inhibitors of PP 1/2A have also been shown to induce the
15 activation of NF- κ B in monocytes, Jurkat T cells and Hela cells (Menon *et al.*, 1993; Suzuke *et al.*, 1994) due to the phosphorylation of I κ B α at protein phosphatase 2A-sensitive phosphorylation sites which are different than cytokine-induced phosphorylation sites (Sun *et al.*, 1995). However, according to Baeuerle and colleagues (Schmidt *et al.*, 1995), okadaic acid-mediated activation of NF- κ B in Hela cells requires the induction of oxidative
20 stress. Identification of binding site of NF- κ B in the promoter region of iNOS gene and the activation of NF- κ B during cytokine-induced iNOS expression establishes the role of NF- κ B activation in the induction of iNOS (Xie *et al.*, 1994; Kwon *et al.*, 1995). In contrast to the ability of okadaic acid on the activation of NF- κ B in other cell types (Menon *et al.*, 1993; Suzuke *et al.*, 1994), okadaic acid by itself was unable to induce the activation of NF- κ B in rat
25 primary astrocytes. However, okadaic acid markedly stimulated LPS- or cytokine-mediated activation of NF- κ B in astrocytes. Increase in the activation of NF- κ B in LPS-stimulated astrocytes by okadaic acid paralleled the increase in induction of iNOS indicating that stimulation of iNOS expression in LPS-activated rat primary astrocytes by inhibitors of PP 1/2A is probably mediated via enhanced activation of NF- κ B. However, consistent with the
30 effect of okadaic acid on the activation of NF- κ B in other cell types (Menon *et al.*, 1993; Suzuke *et al.*, 1994), okadaic acid by itself induced the activation of NF- κ B in macrophages but

this activation of NF-kB by okadaic acid did not result in the induction of iNOS indicating that activation of NF-kB by okadaic acid is not sufficient for the induction of iNOS in macrophages. Although similar to astrocytes, okadaic acid stimulated the LPS-mediated activation of NF-kB in rat peritoneal macrophages, yet in sharp contrast to the effect of okadaic acid on the
5 induction of iNOS in astrocytes, the stimulation of NF-kB activation by okadaic acid in LPS-treated macrophages did not parallel with the expression of iNOS. Instead, consistent with a previous report, okadaic acid and other inhibitors of PP 1/2A markedly inhibited LPS- and cytokine-induced expression of iNOS in macrophages. However, the basis for this differential regulation of induction of iNOS in astrocytes and macrophages by inhibitors of PP 1/2A is not
10 understood at the present time.

Earlier, the inventor observed that cAMP-dependent protein kinase A (PKA) also differentially modulates the induction of iNOS in astrocytes and macrophages. Inhibition of the activation of NF-kB and the induction of iNOS with the increase in PKA activity, and stimulation of the activation of NF-kB and the induction of iNOS with the decrease in PP 1/2A
15 activities in astrocytes indicate that both PKA (a serine-threonine protein kinase) and PP 1/2A (serine-threonine phosphoprotein phosphatases) function as inhibitory signals for the induction of iNOS in astrocytes modulating different steps of the signal transduction pathways. In contrast, in macrophages, inhibitors of PKA inhibited the LPS-mediated activation of NF-kB and induction of iNOS, and inhibitors of PP 1/2A stimulated the LPS-mediated activation of
20 NF-kB but inhibited the induction of iNOS indicating that both PKA and PP 1/2A are necessary components of the LPS-mediated signaling pathways for the induction of iNOS. However, the molecular basis for the differential regulation of activation of NF-kB and expression of iNOS gene by inhibitors of PP 1/2A in rat peritoneal macrophages is not known. In light of the fact that NF-kB is necessary but not sufficient for the expression of iNOS gene and that many of the
25 signal transduction events are cell type specific, the apparent stimulation of NF-kB and inhibition of iNOS gene expression by inhibitors of PP 1/2A clearly delineate that apart from the activation of NF-kB some other signaling pathway(s) sensitive to PP 1/2A is/are responsible for the expression of iNOS gene in macrophages.

The inventor examined the possible involvement of ROS in cytokine-mediated
30 activation of sphingomyelin breakdown and ceramide formation and found that intracellular GSH plays a crucial role in the breakdown of SM to ceramide, in that low GSH levels are

required for ceramide generation and high GSH levels inhibit production of ceramide. Inhibition of cytokine-mediated breakdown of SM to ceramide by antioxidants like N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) and induction of ceramide production by oxidants or pro-oxidants like hydrogen peroxide, aminotriazole, diamide and L-buthione (S,R)-sulfoximine clearly delineate a novel function of ROS and GSH in regulation of the first step of sphingomyelin signal transduction pathway. Moreover, decreased levels of GSH and increased levels of ceramide correlate with the DNA fragmentation in rat primary oligodendrocytes as well as in the banked human brains from patients with neuroinflammatory diseases (*e.g.* multiple sclerosis and X=adrenoleukodystrophy).

Changes in the cellular redox state toward either prooxidant or antioxidant conditions have profound effects on cellular functions. Several lines of evidence presented herein indicate that the first step of cytokine-induced sphingomyelin signal transduction pathway (*i.e.* breakdown of sphingomyelin to ceramide and phosphocholine) is redox sensitive. First, cytokines like TNF- α and IL-1 β decreased intracellular GSH and induced the degradation of sphingomyelin to ceramide in rat primary astrocytes, oligodendrocytes, microglia and rat C₆ glial cells, and pretreatment of the cells with antioxidants like NAC restored the levels of GSH and blocked the degradation of sphingomyelin to ceramide. Second, depletion of endogenous glutathione by diamide or buthione sulfoximine alone induces the degradation of sphingomyelin to ceramide which is blocked by NAC. Third, the increase in intracellular H₂O₂ by the addition of exogenous H₂O₂ or by the inhibition of endogenous catalase by aminotriazole induced the degradation of sphingomyelin to ceramide which is also blocked by NAC. Fourth, besides NAC, pyrrolidine dithiocarbamate (PDTC), an antioxidant but not the precursor of GSH (Laight *et al.*, 1997), also inhibited the TNF- α and IL-1 β -induced hydrolysis of sphingomyelin to ceramide.

Several studies support a role for hydrolysis of sphingomyelin as a stress-activated signaling mechanism in which ceramide plays a role in growth suppression and apoptosis in various cell types including glial and neuronal cells (Brugg *et al.*, 1996; Wiesner and Dawson, 1996). Ceramide activates the proteases of the interleukin converting enzyme (ICE) family, (especially prICE/YAMA/ CPP32), the protease responsible for cleavage of poly-ADP-ribose polymerase (PARP) (Martin *et al.*, 1995) and that the activation of prICE by ceramide and induction of apoptosis are inhibited by overexpression of Bcl-2 (Zhang *et al.*, 1996). Addition

of exogenous ceramides or sphingomyelinase to cells induces stress activated protein kinase (SAPK)-dependent transcriptional activity through the activation of c-jun (Latinis and Koretzky, 1996) and a dominant negative mutant of SEK1, the protein kinase responsible for phosphorylation and activation of SAPK, interferes with ceramide-induced apoptosis
5 (Verheij *et al.*, 1996). These observations also indicate that both Bcl-2 and SAPK function downstream of ceramide in the apoptotic pathway.

The inventor has found that DNA fragmentation and increase in ceramide and decrease in GSH in primary oligodendrocytes and banked human brains with X-ALD and MS clearly indicate that intracellular redox (level of GSH) is an important regulator of apoptosis *via*
10 controlling the generation of ceramide. This conclusion is based on following observations. First, treatment of oligodendrocytes with TNF- α decreased intracellular level of GSH, increased degradation of SM to ceramide and induced DNA fragmentation, however, pretreatment of oligodendrocytes with NAC blocked the TNF- α -mediated decrease in GSH level, increase in ceramide level and increase in DNA fragmentation. Second, treatment of
15 oligodendrocytes only with diamide, a thiol-depleting agent, decreased intracellular level of GSH, increased level of ceramide and induced DNA fragmentation which are prevented by pretreatment of NAC, a thiol-replenishing agent. Third, the inventor found increased fragmentation of DNA in brains from patients with X-ALD and MS where the levels of GSH and ceramide were lower and higher respectively compared to those found in control human
20 brains. These observations clearly indicate that maintenance of the thiol/oxidant balance is crucial for protection against cytokine-mediated ceramide production and thereby against ceramide-induced cytotoxicity.

Recent observation demonstrated that ceramide potentiates the cytokine-mediated induction of inducible nitric oxide synthase (iNOS) in astrocytes and C₆ glial cells. Although
25 ceramide by itself did not induce the expression of iNOS and production of NO, it markedly stimulated the cytokine-induced expression of iNOS and production of NO indicating that sphingomyelin-derived ceramide generation may be an important factor in cytokine-mediated cytotoxicity in neurons and oligodendrocytes in neuroinflammatory diseases. The N-acetyl cysteine (NAC), which has been used to block the cytokine-induced ceramide production in this
30 study and to inhibit cytokine-mediated induction of iNOS is a nontoxic pharmaceutical drug that enters the cell readily and serves both as a scavenger of ROS and a precursor of GSH, the

major intracellular thiol (Smilkstein *et al.*, 1988). Therefore, the use of reductants such as NAC or other thiol compounds, may be beneficial in restoring cellular redox and in inhibition of cytokine-mediated induction of iNOS and breakdown of sphingomyelin thus reducing NO-mediated cytotoxicity as well as ceramide-mediated apoptosis in neuroinflammatory diseases.

Inhibitors, Enhancers and Screening Assays

In still further embodiments, the present invention provides methods for identifying new iNOS and/or proinflammatory cytokine inhibitory compounds, which may be termed as "candidate substances." It is contemplated that such screening techniques will prove useful in the general identification of any compound that will serve the purpose of inhibiting iNOS and/or proinflammatory cytokines, and in preferred embodiments, will provide candidate therapeutic compounds. The present invention also provides methods for identifying new iNOS and/or proinflammatory cytokine stimulatory or enhancing compounds.

It is further contemplated that useful compounds in this regard will in no way be limited to proteinaceous or peptidyl compounds. In fact, it may prove to be the case that the most useful pharmacological compounds for identification through application of the screening assays will be non-peptidyl in nature and, *e.g.*, which will serve to inhibit or enhance iNOS and/or proinflammatory cytokine activity or transcription through a tight binding or other chemical interaction. Candidate substances may be obtained from libraries of synthetic chemicals, or from natural samples, such as rain forest and marine samples.

In preferred embodiments, a candidate induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines is selected from agents that have certain traits or modes of action common to those of the suppressors and/or inhibitors identified herein. Preferred candidate substances would either inhibit the Ras/Raf/MAP kinase pathway, inhibit and/or suppress the induction and/or activation of NF- κ B, inhibit mevalonate synthesis, be an enhancer of protein kinase A, and/or inhibit the farnasylation of proteins, including but not limited to Ras. In certain embodiments the inhibitor of mevalonate synthesis may be an inhibitor of HMG-CoA reductase or suppressor of its induction. In certain aspects the inhibitor of HMG-CoA reductase is a stimulator of AMP-activated protein kinase. In certain other embodiments the inhibitor of of inducible nitric oxide synthase and/or

proinflammatory cytokines may be an inhibitor of mevalonate pyrophosphate decarboxylase or suppressor of its induction. In other embodiments the candidate substance is an antioxidant. In other embodiments the candidate substance is an enhancer of intracellular cAMP. The enhancer of intracellular cAMP may be an inhibitor of cAMP phosphodiesterase and/or
5 suppressor of its induction. In other embodiments the candidate substance is a farnesyl protein transferase inhibitor and/or induction suppressor.

In other preferred embodiments, a candidate induction suppressor and/or inhibitor of inducible nitric oxide synthase and/or proinflammatory cytokines is selected from agents that have certain traits or modes of action common to those of the stimulators and/or enhancers
10 identified herein. For example, a preferred candidate stimulators or enhancers would include a PKA inhibitor.

In other embodiments, the present invention provides methods for identifying new iNOS and/or proinflammatory cytokine inhibitory or stimulatory compounds. To determine whether a candidate substance has inhibitory, suppressor, stimulator, or enhancer activity for
15 iNOS, and/or proinflammatory cytokines, assays may be employed to detect or measure the change in the message, content, and/or activity of iNOS, proinflammatory cytokines such as TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ , proteins involved in second messenger pathways, or transcription factors such as NF-k β .

20 **Nucleic Acid Detection**

Assays for the detection of iNOS, NF-k β , and/or proinflammatory cytokines such as TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ include detection of changes in the amount of nucleic acid message in a cell upon contact with a candidate inhibitor, suppressing agent, enhancer, and/or stimulator. Such assays are described in the specific examples. Additionally,
25 gene sequences for known iNOS, NF-k β , and/or proinflammatory cytokines in a database such as found in the National Center for Biotechnology Information (internet web site: <http://www.ncbi.nlm.nih.gov>) may be used as probes or primers in nucleic acid hybridization embodiments of such assays.

1. Hybridization

The use of a hybridization probe of between 17 and 100 nucleotides in length, or in some aspect of the invention even up to 1-2 Kb or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 20 bases in length are generally preferred, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of particular hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having stretches of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of genes or RNAs or to provide primers for amplification of DNA or RNA from tissues. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating specific genes or detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications, for example, substitution of nucleotides by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, at temperatures
5 ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of
10 being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-
15 containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization, as in PCRTM, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected
20 matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe molecules,
25 hybridization is detected, or even quantified, by means of the label.

2. Amplification and PCRTM

Nucleic acid used as a template for amplification is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic
30 acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be

desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ genes are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each incorporated herein by reference in entirety.

Briefly, in PCRTM, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCRTM amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are
5 described in WO 90/07641, filed December 21, 1990, incorporated herein by reference. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to
10 opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCRTM, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

15 Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

20 An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal
25 amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.
30 Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA

is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

5 Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCRTM-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector
10 moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification
15 systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These
20 amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA
25 polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose
30 a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance

with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a
5 template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at
10 one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

15 Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods
20 include "RACE" and "one-sided PCRTM" (Frohman, 1990, incorporated herein by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product
25 from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There
30 are many kinds of chromatography which may be used in the present invention: adsorption,

partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

All the essential materials and reagents required for changes in iNOS and/or proinflammatory cytokines in a biological sample may be assembled together in a kit. This generally will comprise preselected primers for specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, *etc.*), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such

kits generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair.

In another embodiment, such kits will comprise hybridization probes specific for iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ . Such kits generally will comprise, in
5 suitable means, distinct containers for each individual reagent and enzyme as well as for each marker hybridization probe.

Immunodetection Methods

In still further embodiments, the present invention concerns immunodetection methods
10 for binding, purifying, removing, quantifying or otherwise generally detecting biological components such as iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ components. The antibodies specific for iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ may be prepared in accordance with the present invention may be employed to detect wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ proteins, polypeptides or
15 peptides. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura *et al.* (1987), incorporated herein by reference.

In general, the immunobinding methods include obtaining a sample suspected of containing an iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein, polypeptide or peptide, and contacting the sample with a first anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6,
20 IL-8 and/or IFN- γ antibody in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

These methods include methods for purifying wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ proteins, polypeptides or peptides as may be employed in purifying wild-type or mutant iNOS, NF-k TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or
25 IFN- γ proteins, polypeptides or peptides from patients' samples or for purifying recombinantly expressed wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ proteins, polypeptides or peptides. In these instances, the antibody removes the antigenic wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein, polypeptide or peptide component from a sample. The antibody will preferably be linked to a
30 solid support, such as in the form of a column matrix, and the sample suspected of containing the wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antigenic

component will be applied to the immobilized antibody. The unwanted components will be washed from the column, leaving the antigen immunocomplexed to the immobilized antibody, which wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antigen is then collected by removing the wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ from the column.

The immunobinding methods also include methods for detecting or quantifying the amount of a wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein reactive component in a sample, which methods require the detection or quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing a wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein, polypeptide or peptide, and contact the sample with an antibody against wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ , and then detect or quantify the amount of immune complexes formed under the specific conditions.

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing a wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein-specific antigen, such as a lymphocytes, macrophages, endothelial cells, astrocytes, microglia, oligodendrocytes and/or neuron tissue section or specimen, a homogenized lymphocytes, macrophages, endothelial cells, astrocytes, microglia, oligodendrocytes and/or neuron tissue extract, or even any biological fluid that comes into contact with diseased lymphocytes, macrophages, endothelial cells, astrocytes, microglia, oligodendrocytes and neurons tissue, including blood and serum, although tissue samples and extracts are preferred.

Contacting the chosen biological sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time lone enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological or enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837;
5 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The iNOS, NF- κ B, TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibody employed in
10 the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a
15 detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled
20 secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the
25 secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is
30 desired.

In the detection of an alteration in the levels of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ gene message, translation product, and/or activity in or from a biological sample, such as a cell, tissue, or organism, a comparison is made between a biological sample upon contact with a candidate suppressor, inhibitor, stimulator, and/or enhancer, to that of a similar or like biological sample that has not contacted with a candidate suppressor, inhibitor, stimulator, and/or enhancer. Reduced levels of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ gene message, translation product, and/or activity of a cell or patient is indicative of the candidate substance being a inhibitor or suppressor. An enhancer or stimulator would be identified by increased levels of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ gene message, translation product, and/or activity. Preferably, the biological sample has been contacted with a known inducer or enhancer, such as LPS and/or proinflammatory cytokines, or a suppressor or inhibitor, either before, during, and/or after contact with the candidate substance, to help measure the candidate substance's effect on the activity of the known inducer, suppressor, inhibitor, or enhancer. Those of skill in the art are very familiar with differentiating between significant differences in types or amounts of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ , which represent a positive identification, and low level or background changes of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ . Indeed, background expression levels are often used to form a "cut-off" above which increased detection will be scored as significant or positive. In this case, "background" levels may be the levels of iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ seen after stimulation of a cell or patient with endotoxin and/or a cytokine, preferably a proinflammatory cytokine.

1. ELISAs

As detailed above, immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, the anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibodies are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein antigen, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein antigen may be detected. Detection is generally achieved by the addition of another anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples suspected of containing the wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antigen are immobilized onto the well surface and then contacted with the anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibodies are detected. Where the initial anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first anti- iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ proteins, polypeptides or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies against wild-type or mutant iNOS, NF-k β , TNF- α , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein are added to the wells, allowed to bind, and detected by means of their label. The amount of wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies against wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ before or

during incubation with coated wells. The presence of wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein in the sample acts to reduce the amount of antibody against wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein available for binding to the well and thus reduces the ultimate signal. This is also appropriate for detecting antibodies against wild-type or mutant iNOS, NF-k β , TNF- α , IL-1 β , IL-2, IL-6, IL-8 and/or IFN- γ protein in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described below.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween.

These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours or so, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at about 4°C or so.

5 Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

10 To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and
15 under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-
20 sulfonic acid (ABTS) and H₂O₂, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

2. Immunohistochemistry

25 The antibodies of the present invention may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990;
30 Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

Second Generation Inhibitors or Enhancers

In addition to the inhibitory compounds initially identified, the inventor also contemplates that other sterically similar compounds may be formulated to mimic the key portions of the structure of the inhibitors and/or enhancers. Such compounds, which may include peptidomimetics of peptide inhibitors and/or enhancer, may be used in the same manner as the initial inhibitors and/or enhancers.

Certain mimetics that mimic elements of protein secondary structure are designed using the rationale that the peptide backbone of proteins exists chiefly to orientate amino acid side chains in such a way as to facilitate molecular interactions. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

Some successful applications of the peptide mimetic concept have focused on mimetics of β -turns within proteins, which are known to be highly antigenic. Likely β -turn structure within a polypeptide can be predicted by computer-based algorithms, as is well known to the skilled artisan. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

The generation of further structural equivalents or mimetics may be achieved by the techniques of modeling and chemical design known to those of skill in the art. The art of computer-based chemical modeling is now well known. Using such methods, a chemical that

specifically inhibits iNOS and/or proinflammatory cytokines can be designed, and then synthesized, following the initial identification of a compound that inhibits iNOS and/or proinflammatory cytokines activity and/or induction, but that is not specific or sufficiently specific to inhibit iNOS activity in individuals suffering from demyelinating diseases or neural trauma. Also using such methods, a chemical that specifically enhances iNOS and/or proinflammatory cytokines can be designed, and then synthesized, following the initial identification of a compound that enhances iNOS and/or proinflammatory cytokines activity and/or induction. It will be understood that all such sterically similar constructs and second generation molecules fall within the scope of the present invention.

Optimization in Therapy

A compound identified as having the ability to inhibit or enhance the induction of iNOS and/or cytokines can be assayed its optimum therapeutic dosage alone or in combination with another anti-iNOS, anti-cytokine or anti-inflammatory agent. Such assays are well known to those of skill in the art, and include tissue culture or animal models for various disorders that are treatable with such agents.

Examples of such assays include those described herein and in U.S. Pat. No. 5,696,109, the disclosure of which is incorporated herein by reference in its entirety. For instance, an assay to determine the therapeutic potential of molecules in brain ischemia (stroke) evaluates an agent's ability to prevent irreversible damage induced by an anoxic episode in brain slices maintained under physiological conditions. An animal model of Parkinson's disease involving iatrogenic hydroxyl radical generation by the neurotoxin MPTP (Chiueh *et al.*, 1992, incorporated herein by reference) may be used to evaluate the protective effects of iNOS or pro-inflammatory cytokine induction inhibitors. The neurotoxin, MPTP, has been shown to lead to the degeneration of dopaminergic neurons in the brain, thus providing a good model of experimentally induced Parkinson's disease (*e.g.*, iatrogenic toxicity). An animal model of ischemia and reperfusion damage is described using isolated iron-overloaded rat hearts to measure the protective or therapeutic benefits of an agent. Briefly, rats receive an intramuscular injection of an iron-dextran solution to achieve a significant iron overload in cardiac tissue. Heart are then isolated and then subjected to total global normothermic ischemia, followed by reperfusion with the perfusion medium used initially. During this reperfusion, heart

rate, and diastolic and systolic pressures were monitored. Cardiac tissue samples undergo the electron microscopy evaluation to measure damage to mitochondria such as swelling and membrane rupture, and cell necrosis. Comparison of measured cardiac function and cellular structural damage with or without the agent or iron-overloading after ischemia/reoxygenation is used to determine the therapeutic effectiveness of the agent. Another assay measures acute lung injury (ALI) in sepsis and endotoxemia. LPS/endotoxin-induced ALI in pigs may be used as a model to measure the effectiveness of an agent for the treatment of sepsis-induced ALI in humans. After infusion of LPS/endotoxin, changes in lung wet-to-dry weight ratio, lung lipid peroxidation, pulmonary arterial hypertension, arterial hypoxemia and decreased dynamic pulmonary compliance is measured to determine the effectiveness of an agent in preventing LPS/endotoxin induced damage.

One of skill in the art will recognize that there are other assays and models for disease states available, including testing in humans. These assays may be used to measure the effectiveness of iNOS and/or pro-inflammatory cytokine induction suppressor and/or inhibitor agent for a particular disease or condition, determine the best agent or combination of agents to be used, and determine the dosages for administration, with routine experimentation.

Combination Therapy

The suppressor agents of the invention may also be used in combination with other therapeutic agents, for example, anti-inflammatory agents, particularly non-steroidal anti-inflammatory drugs (NSAIDs), vasodilator prostaglandins including prostacyclin and prostaglandin E sub 1, cancer chemotherapeutic agents including cisplatin, NO donors or NO inhalation therapy, or PAF--receptor antagonists.

Pharmaceutical Compositions

A further aspect of the invention are compositions comprising a first iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor in a pharmaceutically-acceptable excipient. In a preferred embodiment, the iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), PDTC, 4-phenylbutyrate (4PBA),

5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives thereof.

A further aspect of the invention is a composition which comprises at least two iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor in a pharmaceutically-acceptable excipient. In a preferred embodiment, at least one of the iNOS and/or proinflammatory cytokine inhibitors or suppressors is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), PDTC, 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives thereof.

A further aspect of the invention are compositions comprising a first iNOS and/or proinflammatory cytokine stimulator, enhancer, or inducer in a pharmaceutically-acceptable excipient. A further aspect of the invention is a composition which comprises at least two iNOS and/or proinflammatory cytokine stimulator, enhancer, or inducer in a pharmaceutically-acceptable excipient. In another embodiment, the enhancer, stimulator or inducer of iNOS or proinflammatory cytokines is H-89, myristoylated PKI, (R)-cAMP, forskolin, 8-bromo-cAMP, rolipram and salts, analogs, or derivatives thereof. Inducers, stimulators or enhancers of iNOS and/or proinflammatory cytokines may be tissue specific, and such tissues include microglia cells.

In certain embodiments, the suppressors, inhibitors, stimulators, enhancers and/or inducers of iNOS and/or proinflammatory cytokines may be administered of from about 0.001 mg per kg body weight per day (mg/kg/day) to about 20 mg/kg/day. Of course it is understood that of from about 0.001 mg/kg/day to about 20 mg/kg/day includes doses of from about 0.001, about 0.002, about 0.003, about 0.004, about 0.005, about 0.006, about 0.007, about 0.008, about 0.009, about 0.01, about 0.011, about 0.012, about 0.013, about 0.014, about 0.015, about 0.016, about 0.017, about 0.018, about 0.019, about 0.02, about 0.021, about 0.022, about 0.023, about 0.024, about 0.025, about 0.026, about 0.027, about 0.028, about 0.029, about 0.03, about 0.032, about 0.034, about 0.036, about 0.038, about 0.04, about 0.042, about 0.044, about 0.046, about 0.048, about 0.05, about 0.055, about 0.06, about 0.065, about 0.07, about 0.075, about 0.08, about 0.085, about 0.09, about 0.095, about 0.10, about 0.11, about 0.12, about 0.13, about 0.14, about 0.15, about 0.16, about 0.17, about 0.18, about 0.19, about 0.20,

about 0.21, about 0.22, about 0.23, about 0.24, about 0.25, about 0.26, about 0.27, about 0.28, about 0.29, about 0.30, about 0.31, about 0.32, about 0.33, about 0.34, about 0.35, about 0.36, about 0.37, about 0.38, about 0.39, about 0.40, about 0.41, about 0.42, about 0.43, about 0.44, about 0.45, about 0.46, about 0.47, about 0.48, about 0.49, about 0.50, about 0.51, about 0.52, about 0.53, about 0.54, about 0.55, about 0.56, about 0.57, about 0.58, about 0.59, about 0.60, about 0.65, about 0.70, about 0.75, about 0.80, about 0.85, about 0.90, about 0.95, about 1.00, about 1.05, about 1.10, about 1.15, about 1.20, about 1.25, about 1.30, about 1.35, about 1.40, about 1.45, about 1.50, about 1.55, about 1.60, about 1.65, about 1.70, about 1.75, about 1.80, about 1.85, about 1.90, about 1.95, about 2.00, about 2.10, about 2.20, about 2.30, about 2.40, about 2.50, about 2.60, about 2.70, about 2.80, about 2.90, about 3.00, about 3.10, about 3.20, about 3.30, about 3.40, about 3.50, about 3.60, about 3.70, about 3.80, about 3.90, about 4.00, about 4.10, about 4.20, about 4.30, about 4.40, about 4.50, about 4.60, about 4.70, about 4.80, about 4.90, about 5.00, about 5.25, about 5.5, about 5.75, about 6.00, about 6.25, about 6.5, about 6.75, about 7.0, about 7.25, about 7.5, about 7.75, about 8.00, about 8.25, about 8.5, about 8.75, about 9.0, about 9.25, about 9.5, about 9.75, about 10.00, about 10.5, about 11.0, about 11.5, about 12.00, about 12.5, about 13, about 13.5, about 14.00, about 14.5, about 15, about 15.5, about 16, about 16.5, about 17.0, about 17.5, about 18.00, about 18.5, about 19.0, about 19.5, and about 20.00 mg/kg/day. One may select any dosages described herein as a range of dosage administration, such as a range of about 1.45 mg/kg/day to about 11 mg/kg/day, or about 0.24 mg/kg/day to about 14 mg/kg/day, etc., as well as any values within such ranges that are not specifically recited.

One of skill in the art will recognize that the toxicity for different suppressors, inhibitors, enhancer, stimulator and/or inducers of iNOS and/or proinflammatory cytokines either alone, in combination with each other, or in combination with other pharmaceuticals may limit the maximum dose administered to a patient. Dosage optimization for maximum benefits with minimal toxicity in a patient may be optimized by those of skill in the art without undue experimentation using any method to determine optimum dosage in a patient as is known to those of the art, or using the methods described herein. Additionally, the suppressors, inhibitors, enhancer, stimulator and/or inducers of the present invention may be obtained from commercial vendors and administered in any of the methods or dosages described in exemplary texts, such as "Remington's Pharmaceutical Sciences" 8th and 15th Editions; the "Physicians'

Desk Reference", 1998 Edition, the Merck Index, 11th Edition, each incorporated herein in their entirety).

In certain preferred embodiments, lovastatin or mevastatin is taken orally with food once daily at about 0.01 mg per kg body weight per day (mg/kg/day) to about 0.24 mg/kg/day.

5 Of course it is understood that about 0.01 mg/kg/day to about 0.24 mg/kg/day includes doses of about 0.01, about 0.02, about 0.03, about 0.04, about 0.05, about 0.06, about 0.07, about 0.08, about 0.09, about 0.10, about 0.11, about 0.12, about 0.13, about 0.14, about 0.15, about 0.16, about 0.17, about 0.18, about 0.19, about 0.20, about 0.21, about 0.22, about 0.23, to about 0.24 mg/kg or so per day. In a preferred embodiment, lovastatin or mevastatin is taken orally with
10 food once daily at about 0.25 mg per kg body weight per day (mg/kg/day) to about 0.55 mg/kg/day. Of course it is understood that about 0.25 mg/kg/day to about 0.55 mg/kg/day includes doses of about 0.25, about 0.26, about 0.27, about 0.28, about 0.29, about 0.30, about 0.31, about 0.32, about 0.33, about 0.33, about 0.34, about 0.35, about 0.36, about 0.37, about 0.38, about 0.39, about 0.40, about 0.41, about 0.42, about 0.43, about 0.44, about 0.45, about
15 0.46, about 0.47, about 0.48, about 0.49, about 0.50, about 0.51, about 0.52, about 0.53, about 0.54, to about 0.55 mg/kg or so per day. When two or more iNOS and/or proinflammatory cytokine inhibitors and/or induction suppressors are administered, the combined dose of two or more iNOS and/or proinflammatory cytokine inhibitors and/or induction suppressors is preferably about 0.25 mg/kg/day to about 0.55 mg/kg/day. It is also specifically contemplated
20 by the inventor that a patient may be treated with about 0.55 mg/kg/day to about 5 mg per day or more of lovastatin and/or mevastatin, including about 0.60, about 0.65, about 0.70, about 0.75, about 0.80, about 0.85, about 0.90, about 0.95, about 1.00, about 1.05, about 1.10, about 1.15, about 1.20, about 1.25, about 1.30, about 1.35, about 1.40, about 1.45, about 1.50, about 1.55, about 1.60, about 1.65, about 1.70, about 1.75, about 1.80, about 1.85, about 1.90, about
25 1.95, about 2.00, about 2.05, about 2.10, about 2.15, about 2.20, about 2.25, about 2.30, about 2.35, about 2.40, about 2.45, about 2.50, about 2.55, about 2.60, about 2.65, about 2.70, about 2.75, about 2.80, about 2.85, about 2.90, about 2.95, about 3.00, about 3.10, about 3.20, about 3.30, about 3.40, about 3.50, about 3.60, about 3.70, about 3.80, about 3.90, about 4.00, about 4.10, about 4.20, about 4.30, about 4.40, about 4.50, about 4.60, about 4.70, about 4.80, about
30 4.90, to about 5.00 mg/kg or more per day. Compositions for such treatment are described in,

for example U.S. Pat. Nos. 3,983,140, and 4,231,938, the disclosures of which are incorporated herein by reference in their entirety.

In yet another preferred embodiment of the invention nitric oxide induced cytotoxicity may be prevented or reduced in a patient by treatment with from about 0.01 mg/kg/day to about
5 2.0 mg/kg/day of rolipram, including about 0.01, about 0.02, about 0.04, about 0.06, about 0.08, about 0.10, about 0.12, about 0.14, about 0.16, about 0.18, about 0.20, about 0.22, about 0.24, about 0.26, about 0.28, about 0.30, about 0.32, about 0.34, about 0.36, about 0.38, about 0.40, about 0.42, about 0.44, about 0.46, about 0.48, about 0.50, about 0.52, about 0.54, about 0.56, about 0.58, about 0.60, about 0.62, about 0.64, about 0.66, about 0.68, about 0.70, about
10 0.72, about 0.74, about 0.76, about 0.78, about 0.80, about 0.82, about 0.84, about 0.86, about 0.88, about 0.90, about 0.92, about 0.94, about 0.96, about 0.98, about 1.00, about 1.01, about 1.02, about 1.04, about 1.06, about 1.08, about 1.10, about 1.12, about 1.14, about 1.16, about 1.18, about 1.20, about 1.22, about 1.24, about 1.26, about 1.28, about 1.30, about 1.32, about 1.34, about 1.36, about 1.38, about 1.40, about 1.42, about 1.44, about 1.46, about 1.48, about
15 1.50, about 1.52, about 1.54, about 1.56, about 1.58, about 1.60, about 1.62, about 1.64, about 1.66, about 1.68, about 1.70, about 1.72, about 1.74, about 1.76, about 1.78, about 1.80, about 1.82, about 1.84, about 1.86, about 1.88, about 1.90, about 1.92, about 1.94, about 1.96, about 1.98, to about 1.00 or more mg/kg/day. Preferably 0.1 mg/kg/day to about 0.7 mg/kg/day is used and most preferably about 0.5 mg/kg/day. Compositions for such treatment have been
20 described in, for example, U.S. Pat. No. 5,672,622, specifically incorporated herein by reference in its entirety.

In yet another preferred embodiment of the invention, nitric oxide induced cytotoxicity may be prevented or reduced in a patient by treatment with about 0.01 mg/kg/day to about 1.0 mg/kg/day of forskolin, including about about 0.01, about 0.02, about 0.04, about 0.06, about
25 0.08, about 0.10, about 0.12, about 0.14, about 0.16, about 0.18, about 0.20, about 0.22, about 0.24, about 0.26, about 0.28, about 0.30, about 0.32, about 0.34, about 0.36, about 0.38, about 0.40, about 0.42, about 0.44, about 0.46, about 0.48, about 0.50, about 0.52, about 0.54, about 0.56, about 0.58, about 0.60, about 0.62, about 0.64, about 0.66, about 0.68, about 0.70, about 0.72, about 0.74, about 0.76, about 0.78, about 0.80, about 0.82, about 0.84, about 0.86, about
30 0.88, about 0.90, about 0.92, about 0.94, about 0.96, about 0.98, and about 1.0 or more

mg/kg/day. Compositions for such treatment have been described in, for example, U.S. Patent No. 5,371,104, incorporated herein by reference in its entirety.

In still yet another preferred embodiment of the invention, nitric oxide induced cytotoxicity may be prevented or reduced in a patient by treatment with about 0.1 mg/kg/day to
5 about 20 mg/kg/day of a farnesyl protein transferase inhibitor, for example, FPT II. Specifically contemplated is any dose within this range, including about 0.1, about 0.5, about 1.0, about 1.5, about 2.0, about 2.5, about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, about 10.0, about 10.5, about 11.0, about 11.5, about 12.0, about 12.5, about 13.0, about 13.5, about
10 14.0, about 14.5, about 15.0, about 15.5, about 16.0, about 16.5, about 17.0, about 17.5, about 18.0, about 18.5, about 19.0, about 19.5 and 20.0 or more mg/kg/day. Preferably about 0.5 mg/kg/day to 10 mg/kg/day is used. Compositions for such treatment are described in, for example, U.S. Pat. No. 5,420,157, specifically incorporated herein by reference in its entirety.

In still yet another preferred embodiment of the invention, nitric oxide induced
15 cytotoxicity may be prevented or reduced in a patient by treatment with up to about 50 mg/kg/day of N-acetyl cysteine. Of course, it will be understood that up to about 50 mg/kg/day includes all dosages described above generically for the iNOS and/or proinflammatory cytokine inhibitors and/or induction suppressors of the present invention, and dosages of from about 20 mg/kg/day to about 50 mg/kg/day, including of from about 20, about
20 20.5, about 21, about 21.5, about 22.0, about 22.5, about 23, about 23.5, about 24, about 24.5, about 25, about 25.5, about 26, about 26.5, about 27, about 27.5, about 28, about 28.5, about 29, about 29.5, about 30, about 30.5, about 31.5, about 32, about 32.5, about 33, about 33.5, about 34, about 34.5, about 35, about 35.5, about 36, about 36.5, about 37, about 37.5, about 38, about 38.5, about 39, about 39.5, about 40, about 40.5, about 41, about 41.5, about 42,
25 about 42.5, about 43, about 43.5, about 44, about 44.5, about 45, about 45.5, about 46, about 46.5, about 47, about 47.5, about 48, about 48.5, about 49, about 49.5, and about 50.0 or more mg/kg/day. Specific compositions for such treatment are disclosed in, for example, U.S. Pat. No. 5,080,960, incorporated herein by reference in its entirety.

Table 1**Contemplated Ranges for Dose Administration in the Methods of the Invention**

Lovastatin	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
	Preferred Range	About 0.01 to about 5.00 mg/kg/day
	More Preferred Range	About 0.25 to about 0.55 mg/kg/day
Mevastatin	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
	Preferred Range	About 0.01 to about 5.00 mg/kg/day
	More Preferred Range	About 0.25 to about 0.55 mg/kg/day
Phenyl Acetic Acid	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
N-acetyl Cysteine	Range	About 0.001 mg/kg/day to about 50.00 mg/kg/day
	Preferred Range	About 0.1 to about 5.0 mg/kg/day
PTDC	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
Forskolin	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
	Preferred Range	About 0.01 to about 1.0 mg/kg/day
Rolipram	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
	Preferred Range	About 0.01 to about 2.0 mg/kg/day
	More Preferred Range	About 0.1 to about 0.7 mg/kg/day
	Even More Preferred Range	About 0.5 mg/kg/day
	Range	
cAMP	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
8-bromo-cAMP	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day

Table 1 - Continued

FTP inhibitor II	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
	Preferred Range	About 0.1 to about 20.0 mg/kg/day
	More Preferred Range	About 0.5 to about 10.0 mg/kg/day
H-89	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
Myristoylated PKI	More Preferred Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
(R)-cAMP	More Preferred Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
(S)-cAMP	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
4-phenylbutyrate (4PBA)	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
5-aminoimidazole- 4-carboxamide ribonucleoside (AICAR)	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
theophylline	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day
papaverine	Range	About 0.001 mg/kg/day to about 20.00 mg/kg/day

The pharmaceutical compositions disclosed herein may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The

percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained. It will be understood to one of skill in the art that the actual amount of active ingredient used may vary depending on a number of variables such as the symptoms of the patient, the size of the patient and the age of the patient.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally *e.g.* intraperitoneally or intravascularly. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria

and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral prophylaxis the polypeptide may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes,

powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or other untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The composition can be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human

administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Therapeutic Kits

5 In one aspect, the invention provides a therapeutic kit comprising, in suitable container means, a therapeutically-effective amount of one or more iNOS inhibitors and/or proinflammatory cytokine inhibitors and/or induction suppressors selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), PDTC, 4-phenylbutyrate (4PBA),
10 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives therefrom, and if desired, a pharmaceutically acceptable excipient. The compositions may be formulated such that they are suitable for oral or parenteral administration.

In another aspect, the invention provides a therapeutic kit comprising, in suitable
15 container means, a therapeutically-effective amount of one or more iNOS inhibitors and/or proinflammatory cytokine inhibitors and/or induction suppressors selected from agents that have certain traits or modes of action common to those of the suppressors and/or inhibitors identified herein. Preferred substances would either inhibit the Ras/Raf/MAP kinase pathway, inhibit and/or suppress the induction and/or activation of NF-kB, inhibit mevalonate synthesis,
20 be an enhancer of protein kinase A, and/or inhibit the farnesylation of proteins, including but not limited to Ras. In certain embodiments the inhibitor of mevalonate synthesis may be an inhibitor of HMG-CoA reductase or suppressor of its induction. In certain aspects the inhibitor of HMG-CoA reductase is a stimulator of AMP-activated protein kinase. In certain other embodiments the inhibitor of of inducible nitric oxide synthase and/or proinflammatory
25 cytokines may be an inhibitor of mevalonate pyrophosphate decarboxylase or suppressor of its induction. In other embodiments the substance is an antioxidant. In other embodiments the substance is an enhancer of intracellular cAMP. The enhancer of intracellular cAMP may be an inhibitor of cAMP phosphodiesterase and/or suppressor of its induction. In other embodiments the substance is a farnesyl protein transferase inhibitor and/or induction suppressor.

30 In other preferred embodiments, a preferred stimulators or enhancers would include a PKA inhibitor.

The other preferred embodiments, the inhibitors, suppressors stimulators or enhancers would be identified by the screening assay described herein.

The diagnostic/therapeutic kits comprising the pharmaceutical compositions disclosed herein will generally contain, in suitable container means, a therapeutically-effective amount of an iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor in a pharmaceutically acceptable excipient. The kit may have a single container means that contains the iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor and a suitable excipient or it may have distinct container means for each compound.

The components of the kit may be provided as liquid solution(s), or as dried powder(s).

When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The iNOS and/or proinflammatory cytokine inhibitor(s) and/or induction suppressor(s) may also be formulated into a syringeable composition. In which case, the container means may itself be a syringe, or other such like apparatus, from which the formulation may be administered into the body, preferably by injection or even mixed with the other components of the kit prior to injection. The iNOS and/or proinflammatory inhibitor and/or induction suppressor to be administered may be a single inhibitor, or a composition comprising two or more inhibitors in a single or multiple dose for administration. Alternatively, one or more inhibitors may be administered consecutively or concurrently with other agents as deemed appropriate by the clinician. Dosage of each of the compositions will vary from subject to subject depending upon severity of conditions, size, body weight, *etc.* The calculation and adjustment of dosages of pharmaceutical compositions is well-known to those of skill in the art.

In an alternate embodiment, components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor may be placed, preferably, suitably allocated. Where two or more inhibitors and/or suppressors are provided, the kit will also generally contain a second vial or other container into which this additional inhibitors and/or suppressors may be formulated. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained. Alternatively, the vials may be prepared in such a way as to permit direct introduction of the composition into an intravenous drug delivery system.

Irrespective of the number or type of containers, the kits of the invention may also comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

The term "iNOS and/or proinflammatory cytokine inhibitor and/or induction suppressor" and also includes derivatives of the compounds disclosed herein which exhibit at least some biological activity in common with the unmodified compound. In general these compounds are inhibitors of inducible nitric oxide synthase and/or proinflammatory cytokines.

The following examples are included to demonstrate new and inventive methods of the inventor and preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Inhibition of iNOS and Cytokines

Reagent Recombinant rat IFN- γ , DMEM/F-12 medium, RPMI 1640 medium, fetal bovine serum and Hanks' balanced salt solution (HBSS) were obtained from GIBCO. Human IL1- β was obtained from Genzyme, USA. Mouse recombinant TNF- α was obtained from Boehringer Mannheim, Germany. Lovastatin, mevastatin and farnesyl pyrophosphate were obtained from Biomol, USA and CalBiochem, USA. Cholesterol, ubiquinone, arginase, N-Acetyl cystein (NAC), pyrrolidine dithiocarbamate (PDTC), NADPH, FAD, tetrahydrobiopterin, Dowen-50W and LPS (*Escherichia coli*) were obtained from Sigma (St. Louis, MO). N^G-methyl-L-arginine (L-NMA), FPT inhibitor II and antibodies against mouse macrophage iNOS were obtained from Calbiochem, USA. Immunoassay kits for TNF- α , IL-1 β and IL-6 were obtained from R&D, USA. NF-k β DNA binding protein detection kit was obtained from GIBCO/BRL, USA. [γ -³²P]ATP (3000 Ci/mmol) and L-[2,3,4,5-³H] arginine were obtained from Amersham, USA. Sodium salt of phenylacetic acid (NaPA) was prepared from phenylacetic acid (Sigma) and NaOH as described (Samid *et al.*, 1992).

Induction of NO Production in Rat Astrocytes, Microglia and C6 Glial Cells Astrocytes were prepared from rat cerebral tissue as described by McCarthy *et al.* (1980). Cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (FBS). After 10 days in culture, astrocytes were separate from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the complete removal of all oligodendrocytes and microglia, the shaking was repeated twice after a gap of one or two days before subculturing. The microglial contamination was checked by non-specific esterase staining and oligodendrocytes were examined by immunofluorescence using antibodies against GC (McCarthy *et al.*, 1980). Cells were trypsinized, subcultured and stimulated with LPS or different cytokines in serum-free DMEM/F-12 medium.

Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian *et al.* (1986). Briefly, on day 7 to 9 the mixed glial cultures were washed 3 times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37 °C on a rotary shaker. The floating cells were washed and seeded on to plastic tissue culture flasks and incubated at 37°C for 2 h. The attached cells were removed by trypsinization and seeded on to new plates for further studies. Ninety to ninety-five percent of this preparation was found to be positive for

non-specific esterase, a marker for macrophages and microglia. For the induction of NO production, cells were stimulated with LPS or cytokines in serum-free condition.

C6 glial cells, obtained from ATCC, were also maintained and induced with different stimuli as indicated above.

5 *Treatment of Cells With iNOS Inhibitors* Cells in culture were treated with these compounds by addition of these compounds to the cell culture media. Dose ranges are provided in the accompanying figures and tables.

10 *Isolation of Rat Macrophages and Induction of NO Production* Resident macrophages were obtained from rats by peritoneal lavage with sterile RPMI 1640 medium containing 1% fetal bovine serum and 100 µg/ml gentamicin (Wang *et al.*, 1995). Cells were washed three times with RPMI 1640 at 4°C. All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO₂ in air. Macrophages, at a concentration of 2×10⁶/ml in RPMI 1640 medium containing L-glutamine and gentamicin, were added in volumes of 800 µl to a 35 mm plate. After 1 h, nonadherent cells were removed by washing and 800 µl of serum-free
15 RPMI 1640 medium with various stimuli were added to the adherent cells. After 24 h incubation in 5% CO₂ in air at 37°C, the culture supernatants were transferred to measure NO production (Geng *et al.*, 1995; Wang *et al.*, 1995).

20 *Assay of the Viability of Cells Treated with Lovastatin, Mevastatin, NaPA, TNF-α, IL-1β, IFN-γ, or LPS* The cytotoxic effects of the compounds used in various studies disclosed herein were determined by measuring the cell viability by trypan blue exclusion.

It was found that none of the compounds lovastatin, mevastatin, NaPA, TNF-α, IL-1β, IFN-γ, or LPS had a significant effect on the viability of astrocytes, microglia or macrophages. Changes in cell viability can therefore be ruled out as a cause for the disclosed findings.

25 *Assays of NO synthesis and NOS Activity* Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 400 µl of culture supernatant was allowed to react with 200 µl of Griess reagent (Feinstein *et al.*, 1994a; Wang *et al.*, 1995) and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated
30 from a standard curve derived from the reaction of NaNO₂ in the assay. Protein was measured by the procedure of Bradford (1976).

NOS activity was measured directly by production of L-[2,3,4,5-³H]citrulline from L-[2,3,4,5-³H] arginine (Feinstein *et al.*, 1994a). In these studies, 50 µl of macrophage homogenate was incubated at 37°C in presence of 50 mM Tris-HCl (pH 7.8), 0.5 mM NADPH, 5µM FAD, 5µM tetrahydrobiopterin and 12 µM L-[2,3,4,5-³H]arginine (118 mCi/mmol) in a total volume of 200 µl. Assays were carried out for 30 to 40 min and the production of L-[2,3,4,5-³H] citrulline was linear. The reactions were stopped by addition of 800 µl of ice-cold 20 mM HEPES (pH 5.5) followed by addition of 2 ml of Dowex-50W equilibrated in the same buffer. The samples were then centrifuged and the concentration of L-[³H]citrulline was determined in the supernatant by liquid scintillation counting. Protein was measured by the procedure of Bradford (1976).

Immunoblot analysis for iNOS Following 24 h incubation in the presence or absence of stimuli by different cytokines or LPS, macrophages were scraped off, washed with Hank's buffer, and homogenized in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1mM PMSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin). After electrophoresis the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and [¹²⁵I]-labeled protein A (Singh *et al.*, 1988).

Cells pre-incubated in serum-free media with different concentrations of lovastatin (5 or 10 µM) or NaPA (2 or 5 mM) or a combination of 2 µM lovastatin and 2 mM NaPA for 8 h received 1.0 µg/ml of LPS. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described in Example 7. Western blot analysis for iNOS protein of LPS-stimulated astrocytes clearly showed that both lovastatin and NaPA significantly inhibited the LPS-mediated induction of iNOS protein. A combination of lovastatin and NaPA at dose lower than the one used individually almost completely inhibited LPS-induced production of NO and expression of iNOS.

RNA isolation, Northern blot analysis, and reverse-transcriptase coupled polymerase chain reaction (RT-PCR) Stimulated peritoneal macrophages were taken out from culture dishes directly by adding Ultraspec-II RNA reagent (Biotech Laboratories Inc.) and total RNA was isolated according to the manufacturer's protocol. For northern blot analyses, 20 µg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels,

electrotransferred to Hybond-Nylon Membrane (Amersham) and hybridized at 68°C with ³²P-labeled cDNA probe using Express Hyb hybridization solution (Clontech) as described by the manufacturer. The cDNA probe was made by PCRTM amplification using two primers (forward primer: 5'-CTCCTTCAAAGAGGCAAAAATA-3' (SEQ ID NO:1); reverse primer: 5'-CACTTCCTCCAGGATGTTGT-3' (SEQ ID NO:2)) (Geller *et al.*, 1993). After hybridization filters were washed two to three times in solution I (2× SSC, 0.05% SDS) for 1h at room temperature followed by solution II (0.1× SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed to X-ray film (Kodak). The same filters were stripped and rehybridized with probes for GAPDH. The relative mRNA content for iNOS was measured after scanning the bands with a Biorad (Model GS-670) imaging densitometer.

Five micrograms of total RNA was reverse transcribed by using oligo-dT by using 1 mM of each dNTP, 40 U of RNase inhibitor (Promega), 50 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Stratagene) and reverse transcription buffer (Stratagene) in a 50 µl reaction volume. The integrity of the RNAs was checked by running an alkaline RNA gel. The first strand cDNA synthesis was carried out at 37°C for 1 h. To check the quality of cDNAs for iNOS (730 bp) and for GAPDH (528 bp), the same cDNA was used as a control to amplify 1.5 kb fragment of a iNOS gene. Five microliters of this 1st strand cDNA was used to amplify by PCRTM in 100 µl reaction volume containing 0.2 µM of each primer, 200 µM of each dNTP, manufacturer-supplied 1X buffer containing 1.5 mM MgCl₂ and 2.5 U of Taq DNA polymerase (Stratagene). A total of 30 cycles were run with each cycle having denaturation at 91°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min. Final extension was carried out 72°C for 10 min. Oligonucleotide primers for iNOS (forward primer: 5'-CTCCTTCAAAGAGGCAAAAATA-3' (SEQ ID NO:1); reverse primer: 5'-CACTTCCTCCAGGATTGGTG-3' (SEQ ID NO:3)) were synthesized based on the sequences described by Geller *et al.* (1993). The PCRTM amplified products were further confirmed by restriction mapping. Oligonucleotide primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: 5'-ACCACCATGGAGAAGGCTGG-3' (SEQ ID NO:4); reverse primer: 5'-CTCAGTGTAGCCCAGGAT GC-3' (SEQ ID NO:5)) were used as control. PCRTM products were visualized by electrophoresis in 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide and photographed with a DS-34 type camera. The relative mRNA

content for iNOS was measured after scanning the bands with a Biorad (Model GS-670) imaging densitometer.

After 5 h of incubation, cells were taken out directly by adding ultraspec-II RNA reagent (Biotech Laboratories Inc., Houston, TX) to the plates for isolation of total RNA, and
5 northern blot analysis for iNOS mRNA was carried out as described in Example 7. The Northern blot analysis for iNOS mRNA of LPS-stimulated astrocytes clearly showed that both lovastatin (5 or 10 μ M) and NaPA (2 or 5 mM) significantly inhibited the LPS-mediated induction of iNOS mRNA. A combination of lovastatin and NaPA, at 2 μ M and 2 mM respectively, at a dose lower than the one used individually almost completely inhibited
10 LPS-induced production of NO and expression of iNOS.

Determination of TNF- α , IL-1 β and IL-6 in culture supernatants Macrophages were stimulated with LPS and IFN- γ in serum-free RPMI 1640 media for 24 h in the presence or absence of NAC, PDTC, lovastatin or NaPA, and concentrations of TNF- α , IL-1 β and IL-6 were measured in culture supernatants by using a high-sensitivity enzyme-linked
15 immunosorbent assay (ELISA; Genzyme, Cambridge, MA; R&D Systems, USA) according to the manufacturer's instructions.

Preparation of Nuclear Extracts and Electrophoretic Mobility shift assay Nuclear extracts from stimulated or unstimulated astrocytes (1×10^7 cells) were prepared using the method of Dignam *et al.* (1983) with slight modification. Cells were harvested, washed twice
20 with ice-cold phosphate-buffered saline and lysed in 400 μ l of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin A, and 5 μ g/ml leupeptin (Sigma, St. Louis, MO) containing 0.1% Nondet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μ l of buffer B (20 mM HEPES, pH 7.9, 25% (v/v) glycerol,
25 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin A, and 5 μ g/ml leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μ l of modified buffer C (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and stored at -70°C until use. Nuclear extracts were used
30 for the electrophoretic mobility shift assay using the NF-k β DNA binding protein detection system kit (GIBCO/BRL), according to the manufacturer's protocol.

Inhibition Of LPS- And Cytokine-Induced Production Of NO By Lovastatin To examine whether cytokine-induced NO production is inhibited by lovastatin, rat primary astrocytes were stimulated with different combinations of LPS, TNF- α , IL-1 β and IFN- γ (i.e. LPS + TNF- α , LPS + IFN- γ , TNF- α + IL-1 β , TNF- α + IFN- γ) for 24 h and the production of NO was measured as described above. Cells, pre-incubated in serum-free media with 10 μ M lovastatin for 8 h, received different combinations of LPS and cytokines. Concentration of different stimuli were: LPS, 0.5 μ g/ml; TNF- α , 20 ng/ml; IL-1 β , 50 ng/ml; IFN- γ , 50 U/ml. After 24 h of incubation, the production of nitrite was measured in supernatants. Data was taken as the mean \pm S.D. of three different studies. All the combinations of LPS and cytokines significantly induced the production of NO, however, the addition of 10 μ M lovastatin to astrocytes inhibited the NO production and induction of iNOS protein. Cell homogenates were analyzed for iNOS protein by immunoblotting as described. Under similar conditions lovastatin was also found to inhibit LPS- and cytokine-induced NO production in rat C6 glial cells.

Inhibition of LPS-Induced Expression of iNOS by Lovastatin, NaPA and Mevastatin An examination was made of the effect of lovastatin, mevastatin and mevalonate pyrophosphate decarboxylase (NaPA) on the induction of iNOS and production of NO. As shown in Table 1, it was found that bacterial LPS at a concentration of 1.0 μ g/ml induced the production of NO by about 8-fold. The inhibition of NO production by arginase, an enzyme that degrades the substrate (L-arginine) of NOS and L-NMA, a competitive inhibitor of NOS, indicate that LPS-induced NO production in astrocytes is dependent on NOS-mediated arginine metabolism (Table 2). Lovastatin or mevastatin alone was neither stimulatory nor inhibitory to nitrite production in control astrocytes. However, both the inhibitors, when added 8 h before the addition of LPS, inhibited LPS-mediated induction of nitrite production in astrocytes. Only 25% inhibition in LPS-induced NO production was found when lovastatin was added to the cells along with LPS; however, the degree of inhibition increased with the increase in time of preincubation with lovastatin reaching about 90% inhibition of NO production within 8 to 10 h of preincubation. Lovastatin (5 or 10 μ M) or NaPA (2 or 5 mM) or a combination of 2 μ M lovastatin and 2 mM NaPA also inhibited the induction of NO production in rat primary astrocytes. After 24 h, supernatants were used for nitrite assay as described above. Data was the mean \pm S.D. of three different studies.

Table 2
Inhibition of LPS-induced NO production
in rat primary astrocytes by lovastatin and mevastatin

Stimuli	Nitrite (nmol/mg/24 h)	% Inhibition
Control	2.9 ± 0.5	---
LPS	25.3 ± 3.2	---
LPS + Arginase	5.9 ± 0.8	87
LPS + L-NMA	5.5 ± 0.7	88
Lovastatin	2.9 ± 0.3	---
Mevastatin	2.8 ± 0.4	---
LPS + Lovastatin	5.2 ± 0.5	90
LPS + Mevastatin	5.5 ± 0.5	88

Astrocytes were cultured for 24 h in serum-free DMEM/F-12 with the listed reagents; and nitrite concentration in the supernatants were measured as described. Arginase (100 units/ml) and L-NMA (0.1 mM) were added to the cells together with LPS (1.0 µg/ml). Data are mean ± standard deviation (S.D.) of three different studies.

Inhibition of LPS-Induced Activation of NF-κβ and Expression of iNOS by Lovastatin and NaPA The effect of lovastatin (5 or 10 µM) or NaPA (2 or 5 mM) on LPS-induced activation of NF-κβ in astrocytes was examined by gel-shift DNA-binding assay. Cells incubated in serum-free media received 1.0 µg/ml of LPS. After 1 h of incubation, cells were taken out to prepare nuclear extracts and nuclear proteins were used for the electrophoretic mobility shift assay of NF-κβ as described in Example 10. Lanes were run containing control, LPS, LPS-treated nuclear extract with 25-fold excess of unlabelled probe, and LPS-treated nuclear extract with 50-fold excess of unlabelled probe. Treatment of rat primary astrocytes with 1.0 µg/ml of LPS resulted in the activation of NF-κβ. This gel shift assay detected a specific band in response to LPS that was competitively removed by an unlabelled probe. Lovastatin or NaPA alone at different concentrations failed to induce NF-κβ. However, cells preincubated in serum-free media with lovastatin or NaPA for 8 h were markedly inhibited for

the LPS-induced activation of NF- κ B, indicating that the inhibition of iNOS expression by lovastatin and NaPA is due to the inhibition of NF- κ B.

To evaluate the possible mechanism of the effect of lovastatin and NaPA or to determine whether reduced concentrations of end products as opposed to intermediate products of the mevalonate pathway were responsible for the effects of lovastatin and NaPA, the inventor performed rescue experiments with cholesterol, ubiquinone, mevalonate and farnesyl pyrophosphate (FPP). Cells preincubated in serum-free media with 10 μ M of lovastatin or 5 mM of NaPA for 8 h received 1.0 μ g/ml of LPS along with 100 μ M mevalonate or 200 μ M farnesyl pyrophosphate. After 24 h, supernatants were used for a nitrite assay as described in Example 6. Combinations that were tested included control, LPS alone, LPS + lovastatin, LPS + lovastatin + mevalonate, LPS + lovastatin + FPP, LPS + NaPA, LPS + NaPA + mevalonate, and LPS + NaPA + FPP. Data was measured as the mean \pm S.D. of three different studies. After 5 h of incubation, cells were analyzed for iNOS mRNA by northern blotting technique as described. GAPDH mRNA was also measured. After 1 h of incubation, cells were taken out to prepare nuclear extracts and nuclear proteins were used for the electrophoretic mobility shift assay of NF- κ B as described in Example 10. Addition of 10 μ M ubiquinone or cholesterol to astrocytes did not prevent the inhibitory effect of lovastatin and NaPA. These observations support the conclusion that the depletion of intermediary products rather than end products of mevalonate pathway are responsible for the observed inhibitory effect of lovastatin or NaPA on LPS-induced iNOS expression. On the other hand, mevalonate or FPP substantially reversed the inhibitory effect of lovastatin on iNOS expression and NF- κ B activation. However, FPP not mevalonate reversed the inhibitory effect of NaPA indicating that the utilization of mevalonate rather than its synthesis is the prime target of the NaPA.

Inhibition of LPS-Induced Expression of iNOS in Rat Primary Astrocytes by FPT inhibitor II An examination was made of the effect of FPT inhibitor II, an inhibitor of enzymes that transfers farnasyl group to proteins (e.g. Ras), on LPS-mediated expression of iNOS and activation of NF- κ B in rat primary astrocytes. Cells pre-incubated in serum-free media with 100 μ M or 200 μ M FPT inhibitor II for 1 h received 1.0 μ g/ml of LPS. Samples assayed included control, LPS, LPS + FPT inhibitor II (100 μ M or 200 μ M). After 24 h of incubation, supernatants were used for nitrite assay as described in Example 6. Data was measured as the mean \pm S.D. of three different studies. After 5 h of incubation, cells were analyzed for iNOS

mRNA by northern blotting technique as described. After 1 h of incubation, cells were taken out to prepare nuclear extracts and nuclear proteins were used for the electrophoretic mobility shift assay of NF- κ B as described in Example 10. A preincubation of cells for 1 h with 100 or 200 μ M FPT inhibitor II inhibited LPS-induced activation of NF- κ B, expression of iNOS and production of NO; thus, demonstrating the importance of farnesylation of Ras in LPS-mediated activation of NF- κ B and induction of iNOS in astrocytes.

Lovastatin and NaPA inhibit the LPS-induced expression of Cytokines An examination was made of the effect of NaPA and lovastatin on LPS-induced expression of TNF- α , IL-1 β and IL-6. Rat primary astrocytes pre-incubated in serum-free media with different concentrations of lovastatin (5 or 10 μ M) or NaPA (2 or 5 mM) or a combination of 2 μ M of lovastatin and 2 mM of NaPA for 8 h received 1.0 μ g/ml of LPS. Combinations that were tested included control, LPS, LPS + lovastatin (5 μ M), LPS + lovastatin (10 μ M), NaPA (2 μ M), LPS + NaPA (5 μ M), and LPS + lovastatin (2 μ M) + NaPA (2 μ M). Concentrations of TNF- α , IL-1 β and IL-6 were measured in the supernatants after 24 h of incubation (Table 3) and the mRNA expression of these cytokines was examined in the cells after 5 h of LPS stimulation as described. Bacterial LPS markedly induced the mRNA expression and production of respective cytokines in astrocytes. Although lovastatin or NaPA alone had no effect on the production of cytokines, these two compounds strongly inhibited the LPS-induced production of TNF- α , IL-1 β and IL-6 in the supernatants (Table 3). Additionally, 2 mM NaPA and 2 μ M lovastatin worked more effectively to inhibit LPS-induced production of TNF- α , IL-1 β and IL-6 than 5 mM NaPA or 5 μ M lovastatin alone. The decrease in cytokine production was also accompanied by an inhibition of their mRNA expression demonstrating that lovastatin and NaPA down-regulate the expression of all the inflammatory mediators (iNOS, TNF- α , IL-1 β and IL-6) in astrocytes. No adverse effects on the viability of astrocytes, as measured by trypan blue exclusion, were observed.

Table 3

Inhibition of LPS-induced production of NO, TNF- α , IL-1 β and IL-6 in rat primary astrocytes, microglia and macrophages by lovastatin and NaPA

Cells	Production of NO or cytokines	Treatments		
		LPS only	LPS + Lovastatin	LPS + NaPA
Astrocytes	NO	25.3 \pm 3.2	5.2 \pm 0.4	5.4 \pm 0.6
	TNF- α	5.3 \pm 0.8	0.3 \pm 0.05	0.4 \pm 0.06
	IL-1 β	10.4 \pm 1.5	0.8 \pm 0.1	1.1 \pm 0.2
	IL-6	136.5 \pm 16.8	6.9 \pm 0.9	7.6 \pm 0.8
Microglia	NO	81.2 \pm 6.9	5.9 \pm 0.4	6.9 \pm 0.9
	TNF- α	14.5 \pm 2.1	0.9 \pm 0.1	1.3 \pm 0.2
	IL-1 β	28.2 \pm 3.4	2.1 \pm 0.3	2.4 \pm 0.2
	IL-6	295.6 \pm 33.5	7.8 \pm 1.1	9.3 \pm 1.2
Macrophages	NO	118.5 \pm 12.5	7.2 \pm 0.9	9.5 \pm 0.7
	TNF- α	18.6 \pm 2.3	1.2 \pm 0.1	1.7 \pm 0.2
	IL-1 β	34.6 \pm 4.5	2.3 \pm 0.3	3.1 \pm 0.4
	IL-6	350.0 \pm 27.6	8.3 \pm 0.6	10.2 \pm 1.4

Cells preincubated with 10 μ M lovastatin or 5 mM NaPA for 8 h in serum-free condition was stimulated with 1.0 μ g/ml of LPS. After 24 h of incubation, concentrations of NO, TNF- α , IL-1 β and IL-6 were measured in supernatants as described above. NO is expressed as nmol/24 h/mg protein whereas TNF- α , IL-1 β and IL-6 are expressed as ng/24 h/mg protein. Data are expressed as the mean \pm S.D. of three different experiments.

Inhibition Of LPS-Induced Production Of NO and Cytokines In Rat Primary Microglia And Macrophages By Lovastatin Both macrophages and microglia, important sources of NO and cytokines, actively participate in the pathophysiology of different inflammatory disorders.

- 10 Since lovastatin and NaPA inhibited the LPS-induced production of NO, TNF- α , IL-1 β and IL-6 in astrocytes, a determination of the effect of lovastatin and NaPA on LPS-stimulated production of NO, TNF- α , IL-1 β and IL-6 in rat primary microglia and macrophages was made (Table 3). It was found that the rate of production of NO and cytokines after LPS stimulation

was much higher in both macrophages and microglia than in astrocytes. Similar to astrocytes, lovastatin or NaPA alone had no effect on the production of NO and cytokines in macrophages and microglia. However, both of these compounds strongly inhibited the LPS-induced production of NO, TNF- α , IL-1 β and IL-6 in macrophages and microglia (Table 3). These results demonstrate the importance of these compounds in controlling iNOS produced NO and production of proinflammatory cytokines (TNF- α , IL-1 β and IL-6 and IFN- γ) in microglia and macrophages. It is important to note that under the conditions used, no adverse effects on the viability of microglia or macrophages, as measured by trypan blue exclusion, were observed.

EXAMPLE 2

NAC Inhibits LPS-Induced NO Synthesis In Resident Peritoneal Macrophages

Reagents Reagents were as given in Example 1. Rat macrophages, astrocytes and C6 glial cells were prepared as described in Example 1. Assays to measure the induction of NO synthesis, NOS activity, immunoblot analyses for iNOS, RNA isolation, RT-PCR and determination of TNF- α in culture supernatants were as described in Example 1.

The effect of NAC on LPS-induced NO-synthesis was examined in resident peritoneal macrophages. Resident macrophages were cultured in RPMI medium without serum in presence of different concentrations of LPS and NAC. The concentration of NO as nitrite was measured in cultured supernatants after 24 h. As shown in Table 4, in rat resident macrophages, LPS (1 μ g/ml) induced the production of nitrite, the soluble product of NO in the culture medium (Wang *et al.*, 1995), by more than ten fold. LPS-induced production of nitrite was concentration dependent with maximal induction at 1-5 μ g/ml of LPS (data not shown). NAC itself was neither stimulatory nor much inhibitory to nitrite production in control resident macrophages. However, NAC, when added 2 h before the addition of LPS, inhibited LPS-mediated induction of nitrite production in macrophages. Over 90% inhibition was observed when NAC was used at a concentration of 20 mM. Both L-NMA, a competitive inhibitor of NOS, and arginase suppressed LPS-mediated nitrite secretion, indicating that LPS-induced nitrite release in rat peritoneal macrophages is dependent on NOS-mediated arginine metabolism (Table 4).

Table 4
Inhibition of Arginine-Dependent Nitrite Accumulation in
LPS-Stimulated Resident Macrophages by NAC

Stimuli	Nitrite (nmol/mg Protein ^{24h})	Inhibition (%)
Control	9.8±1.5	
NAC	6.2±0.8	
lps	124.2±9.7	
LPS+NAC	11.6±1.5	91
LPS+NMA	15.8±2.2	87
LPS+arginase	22.5±3.1	82

Resident macrophages were cultured for 24 h in serum-free RPMI 1640 with the listed reagents; nitrite concentration in the supernatants was then measured as described in the methods section. Concentration of reagents were: LPS, 1.0 µg/ml; NAC, 20 mM; NMA, 0.1 mM; arginase, 100 units/ml. NMA and arginase were added to the cells together with LPS whereas NAC was added 2 h before the addition of LPS. Data are mean ± S.D. of three different experiments.

Kinetics of inhibition of NO synthesis by NAC in rat macrophages To determine whether inhibition of LPS-induced NO synthesis by NAC was simply due to delayed induction, nitrite concentrations were measured in LPS-stimulated cultures maintained up to 48 h. Rat resident macrophages were stimulated with 1.0 µg/ml LPS alone or together with 20 mM NAC, where NAC was added 2 h before the addition of LPS. Supernatants were harvested at different time intervals (6 to 48 h) to measure concentrations of nitrite as described. Data was measured as the mean ± S.D. of three different studies. When cells were stimulated in the absence of NAC, nitrite was detected in culture supernatants after 8 h and the concentration of nitrite increased progressively thereafter for 48 h. However, when 20 mM NAC was added 2 h before the addition of LPS, nitrite production was significantly inhibited. From the onset of detectable NO release until 24 h, nitrite accumulated at a rate of 5.2 nmol/mg/h in the absence of NAC and at 0.5 nmol/mg/h in the presence of NAC.

To study the Effect of decreasing or increasing the time interval between addition of LPS and NAC on LPS-stimulated macrophage NO production, resident macrophages were incubated with 1.0 µg/ml LPS. NAC (20 mM) was added to cultures 2 h or 1 h before, simultaneously or 1 h, 3 h, 5 h, or 7 h after the addition of LPS. Supernatants were collected 24 h after the addition of LPS. Each value was determined as the mean \pm S.D. of three different studies. Culture supernatants were collected after 24 h of incubation to measure the concentration of nitrite. Maximal suppression of nitrite production was observed when NAC was added 2 h before the addition of LPS. When NAC was added after the addition of LPS, the extent of inhibition progressively decreased. Only 20% inhibition was observed when NAC was added 7 h after the addition of LPS, indicating that inhibition of nitrite production by NAC is due to the inhibition of oxygen radical-mediated signaling reactions.

NAC and PDTC inhibit LPS-mediated induction of iNOS in rat resident macrophages

To understand the mechanism of inhibition of LPS-induced nitrite production in resident macrophages by antioxidants, an examination was made of the effect of NAC and PDTC on the formation of L-citrulline from L-arginine, the reaction which is catalyzed by NOS in homogenates of macrophages. Homogenates were prepared from macrophages that had been incubated for 24 h with 1.0 µg/ml LPS in the presence or absence of 20 mM NAC. NAC was added to the cells 2 h before the addition of LPS. Production of L-[2,3,4,5-³H]citrulline from L-[2,3,4,5-³H]arginine was determined at different time points. Data was determined as the mean of two separate studies. The formation of L-citrulline from L-arginine was linear up to 40 min in LPS-activated macrophages in the absence of NAC whereas in the presence of NAC, formation of L-citrulline was strongly inhibited.

In another study, cells received different concentrations of NAC and PDTC, 2 h before the addition of 1.0 µg/ml LPS. Samples that were tested included control, LPS, 5 mM NAC + LPS, 10 mM NAC + LPS, 20 mM NAC + LPS, 50 µM PDTC + LPS, and 100 µM PDTC + LPS. After 24 h of incubation, cells were washed, scraped off, and homogenized. NOS activity was measured in cell homogenates as described. Data was determined as the mean \pm S.D. of three different studies. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described. After 6 h of incubation, cells were taken out from culture dishes directly by adding ultraspec-II RNA reagent (Biotechx Laboratories Inc.). Total RNA of each sample was prepared,

reverse-transcribed and amplified by using specific primers for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. PCRTM products were electrophoresed in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and photographed with a DS-34 type polaroid camera.

- 5 Both NAC and PDTC inhibited LPS-induced NOS activity as evidenced by L-citrulline formation, and PDTC at a concentration of 100 µM was as potent as NAC at 20 mM. Immunoblot analysis with antibodies against murine macrophage iNOS and RT-PCR for iNOS mRNA analysis of LPS-stimulated macrophages incubated in the presence or absence of NAC or PDTC show that both the antioxidants inhibited LPS-mediated induction of iNOS protein and mRNA, indicating that LPS induced induction of iNOS protein in macrophages via oxygen radicals signal pathway.

- Inhibition Of LPS- And/Or Cytokine-Induced NO Production By NAC* Peritoneal macrophage iNOS can be induced not only by LPS but also by IFN-γ, in combination with either IL-1β or TNF-α (Mehta *et al.*, 1994). To determine whether cytokine-induced NO synthesis is also inhibited by NAC, resident macrophages were cultured with TNF-α, IL-1β or IFN-γ separately or in several combinations, in the presence or absence of NAC. IL-1β or TNF-α when added alone was not able to induce nitrite production; whereas, IFN-γ alone significantly increased NOS-mediated nitrite production (Table 5). Additionally, different combinations of cytokines and LPS induced high level of nitrite production and NOS activity (Table 5). However, NAC, when added 2 h before the addition of cytokines, potentially inhibited the induction of nitrite production. This inhibition of nitrite production was associated with the inhibition of NOS activity as measured by the formation of L-citrulline (Table 5).

- NAC and PDTC inhibit LPS/cytokines stimulated induction of iNOS in rat macrophages*
- 25 Cells received 20 mM NAC 2 h before the addition of different cytokines. After 24 h of incubation with different cytokines, cells were scraped off, washed, and homogenized. Homogenates were immunoblotted with antibodies against mouse macrophage iNOS as described in Example 7. After 6 h of incubation with different stimuli, cells were taken out from culture dishes directly by adding ultraspec-II RNA reagent (Biotecx Laboratories Inc.).
- 30 Total RNA of each sample was prepared, reverse-transcribed, and amplified by using specific primers for iNOS and GAPDH mRNA. PCRTM products were electrophoresed in 1.5% agarose

gel containing 0.5 μ g/ml ethidium bromide and photographed with a DS-34 type polaroid camera. Bands were scanned with a Biorad (Model GS-670) imaging densitometer. The ratio of iNOS gene product to the internal standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to quantitate the message level. Results were measured as the mean \pm S.D. of three different studies. Assays were done for control, LPS + TNF- α , LPS + IL-1 β , LPS + IFN- γ , TNF- α + IL-1 β , TNF- α + IFN- γ , NAC + LPS + TNF- α , NAC + LPS + IL-1 β , NAC + LPS + IFN- γ , NAC + TNF- α + IL-1 β , and NAC + TNF- α + IFN- γ . Concentrations of different stimuli were 0.2 μ g/ml for LPS, 50 U/ml for IFN- γ , 20 ng/ml for TNF- α , and 25 ng/ml for IL-1 β . The LPS and cytokine combinations stimulated nitrite production, while NAC in combination with LPS and/or cytokines inhibited nitrite production. This inhibition was associated with the inhibition of NOS activity as measured as well as the inhibition of expression of the iNOS protein and mRNA, indicating with the results described above for Table 5 that LPS- and cytokines-mediated expression of iNOS involves oxygen radicals signal pathway.

Table 5

**Inhibition nitrite production and NOS activity
by NAC in stimulated rat resident macrophages**

Stimuli	NAC (20mM)	Nitrite (nmol/mg/24h)	Inhibition (%)	L-citrulline (pmol/min/mg)	Inhibition (%)
Control	-	9.8 \pm 1.5		13.5 \pm 1.8	
	+	6.2 \pm 0.8	37	10.2 \pm 0.9	25
LPS	-	124.2 \pm 9.7		156.4 \pm 21.2	
	+	11.6 \pm 1.5	91	14.2 \pm 2.3	91
IL-1 β	-	21.4 \pm 1.9		22.5 \pm 3.2	
	+	9.2 \pm 0.7	57	12.9 \pm 2.1	43
TNF- α	-	27.9 \pm 3.5		32.8 \pm 2.9	
	+	10.1 \pm 1.6	64	12.9 \pm 2.3	61
IFN- γ	-	87.9 \pm 10.2		132.4 \pm 11.6	
	+	9.9 \pm 1.8	89	13.2 \pm 2.4	90

Table 5 - Continued

Stimuli	NAC (20mM)	Nitrite (nmol/mg/24h)	Inhibition (%)	L-citrulline (pmol/min/mg)	Inhibition (%)
TNF- α + IL-1 β	-	136.9 \pm 15.8		182.6 \pm 20.2	
	+	22.8 \pm 2.7	83	28.2 \pm 4.3	85
TNF- α + IFN- γ	-	145.9 \pm 17.4		201.6 \pm 22.5	
	+	29.6 \pm 4.2	79	36.9 \pm 3.4	82
LPS + TNF- α	-	168.2 \pm 19.4		232.7 \pm 20.6	
	+	13.6 \pm 2.6	92	19.7 \pm 2.7	91
LPS + IFN- γ	-	162.3 \pm 15.2		218.5 \pm 23.6	
	+	25.2 \pm 3.9	84	32.3 \pm 4.6	83
LPS + IL-1 β	-	149.6 \pm 11.2		204.3 \pm 17.6	
	+	28.4 \pm 3.7	81	44.9 \pm 6.1	78

Nitrite accumulation in the supernatants and NOS activity in the cells were measured as described. When different stimuli were used alone, their concentrations were: LPS, 1.0 μ g/ml; TNF- α , 100 ng/ml; IL-1 β , 200 ng/ml; IFN- γ , 200 U/ml. When stimuli were used in different combinations their concentrations were: LPS, 0.2 μ g/ml; TNF- α , 20 ng/ml; IL-1 β , 25 ng/ml; IFN- γ , 50 U/ml. Cells received NAC 2 h before the addition of different stimuli. Data are mean \pm S.D. of three different experiments.

NAC Inhibits LPS And/Or Cytokine-Mediated NO Production In Rat Astrocytes And C6 Glial Cells A study was carried out to determine if NAC inhibits LPS and/or cytokine-mediated NO production in rat astrocytes and glial cells. Both astrocytes and C6 glial cells are reported to express iNOS in the presence of different stimuli (Feinstein *et al.*, 1994a; Hu *et al.*, 1995). Similar to previous reports (Feinstein *et al.*, 1994a; Feinstein *et al.*, 1994b), incubation of C6 cells with LPS, TNF- α , IFN- γ or IL-1 β alone did not stimulate nitrite production whereas addition of several combinations of either LPS or cytokines induced the production of NO (Table 6). In contrast to the induction of NO production found in C6 glial cells, either LPS or cytokines alone induced the production of NO in cultured rat astrocytes (Table 6). However, similar to macrophages, addition of NAC at 20 mM concentration 2 h

prior to the addition of several cytokines blocked the induction of NO production in both C6 glial cells and astrocytes (Table 6).

Table 6
Inhibition of nitrite production by NAC
in stimulated astrocytes and C6 glial cells

Stimuli	NAC	Astrocytes		C6 glial cells	
		Nitrite (nmol/mg/24h)	Inhibition (%)	Nitrite (nmol/mg/24h)	Inhibition (%)
Control	-	4.3 ± 1.2		3.1 ± 0.4	
	+	-----	-----	-----	-----
LPS	-	27.8 ± 5.2		3.5 ± 0.4	
	+	5.6 ± 1.6	80	-----	-----
TNF- α	-	23.4 ± 3.9		3.6 ± 0.2	
	+	10.5 ± 2.3	55	-----	-----
IL-1 β	-	30.1 ± 2.6		3.8 ± 0.5	
	+	7.8 ± 1.8	74	-----	-----
IFN- γ	-	22.3 ± 4.2		5.3 ± 1.1	
	+	4.4 ± 1.2	80	-----	-----
LPS + TNF- α	-	41.8 ± 2.8		33.9 ± 4.2	
	+	15.2 ± 1.5	64	7.1 ± 1.1	79
LPS + IL-1 β	-	21.7 ± 2.9		22.8 ± 3.4	
	+	4.5 ± 2.8	79	5.8 ± 1.2	75
LPS + IFN- γ	-	50.7 ± 6.8		32.1 ± 4.2	
	+	23.5 ± 1.4	54	8.2 ± 1.4	74
TNF- α + IL-1 β	-	36.4 ± 4.5		20.7 ± 3.1	
	+	5.2 ± 0.7	86	4.2 ± 0.8	80
TNF- α + IFN- γ	-	38.4 ± 2.6		30.9 ± 5.1	
	+	14.1 ± 2.04	63	8.4 ± 2.1	73

Astrocytes and C6 glial cells were cultured for 24 h in serum-free DMF-12 medium with the listed stimuli, and nitrite accumulation in the supernatants was measured as described

previously. Concentrations of different stimuli were the same as described in the legend of Table 4. Data are mean \pm S.D. of three different experiments.

Inhibition of LPS-mediated TNF- α production by NAC and PDTC in macrophages LPS stimulates a variety of cell types including macrophages to induce the production of TNF- α . To study whether antioxidants effect the production of TNF- α , rat resident macrophages were cultured in serum-free RPMI 1640 medium. They were either treated with antioxidants (NAC and PDTC) 2 h before the addition of LPS or IFN- γ alone or in combination (Table 7). LPS alone at a concentration of 1.0 μ g/ml induced appreciable amounts of TNF- α production (20.3 \pm 3.6 ng/mg/24 h) and the addition of IFN- γ augments the action of LPS (Table 7). However, IFN- γ alone, was ineffective in inducing TNF- α from macrophages. Pre-incubation of cells with either NAC or PDTC almost completely eliminated the induction of TNF- α production by LPS and IFN- γ . These results indicate that similar to the induction of iNOS, the production of TNF- α by LPS and IFN- γ involves oxygen radicals signal pathway.

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Table 7
Effect of NAC and PDTC on TNF- α production in rat resident
macrophages stimulated with LPS and IFN- γ

Reagents	TNF- α (ng/24 h/mg protein)	Inhibition (%)
Control	0.7 \pm 0.1	
NAC	0.6 \pm 0.2	
PDTC	0.7 \pm 0.2	
LPS	20.3 \pm 3.6	
IFN- γ	1.3 \pm 0.4	
LPS + IFN- γ	31.6 \pm 2.9	
LPS + NAC	1.6 \pm 0.3	92
LPS + PDTC	0.9 \pm 0.1	95
LPS + IFN- γ + NAC	2.3 \pm 0.2	93
LPS + IFN- γ + PDTC	1.4 \pm 0.3	96

Resident macrophages cultured in serum-free RPMI 1640 medium received 20 mM NAC or 100 μ M PDTC 2 h before the addition of stimuli. When stimuli were used separately their concentrations were: LPS, 1.0 μ g/ml; IFN- γ , 200 U/ml, and when they were used together, their concentrations were: LPS, 0.2 μ g/ml; IFN- γ , 50 U/ml. Supernatants were collected 24 h after the addition of stimuli to measure TNF- α concentration using ELISA as described.

EXAMPLE 3

Modulation of LPS-induced NO Production and Expression of iNOS in Rat Primary Astrocytes by Compounds That Modulate Intracellular Levels of cAMP

The activation of PKA correlates with the inhibition of LPS-induced iNOS expression in rat primary astrocytes. Primary astrocytes in serum-free DMEM/F-12 received 10 μ M forskolin, 500 μ M 8-Br-cAMP, 5 μ M (S_p)-cAMP, 0.2 μ M H-89, or 20 μ M (R_p)-cAMP 15 min before the addition of 1.0 μ g/ml LPS. Nitrite concentrations were measured in supernatants after 24 h; and NOS activities were measured in cell homogenates as described herein. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS. After 6 h of incubation, cells were taken out from culture dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc.) to isolate total RNA, and Northern blot analyses for iNOS mRNA were carried out as described in Examples 7 and 8. After 30 min of incubation, PKA activities were measured in cells by phosphorylation of Kemptide in the presence or absence of the inhibitor peptide PKI. Results were determined as the mean \pm S.D. of three different studies. Assay were conducted for control, LPS, LPS + forskolin, LPS + 8-bromo-cAMP, LPS + (S_p)-cAMP, LPS + H-89, and LPS + (R_p)-cAMP.

The compounds forskolin, 8-bromo-cAMP, and (S_p)-cAMP, known to increase intracellular cAMP, inhibited the LPS-stimulated NO production as nitrite, iNOS activity as conversion of arginine to citrulline, expression of iNOS protein and iNOS mRNA, and activated PKA activity. The inactive forskolin analogue, 1,9-dideoxyforskolin (10 μ M), neither inhibited the LPS induced iNOS activity nor stimulated the PKA activity (Table 8). Other PKA activators like β -adrenergic receptor agonist, isoproterenol (10 μ M), and cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (1 mM), also inhibited LPS-stimulated NO production and iNOS activity (Table 8). On the other hand,

LPS-stimulated NO production, iNOS activity, and expression of iNOS protein and mRNA were increased by PKA inhibitors (H-89 and (R_p)-cAMP). However, in the absence of LPS neither PKA activators nor PKA inhibitors had any effect on the production of NO. This inhibition of NO production by cAMP was not only confined to astrocytes, but forskolin was also found to inhibit LPS, and cytokine-induced NO production in rat C₆ glial cells. C₆ glial cells incubated in serum-free DMEM/F-12 received 10 μ M forskolin 15 min before the addition of LPS and cytokines. Nitrite concentrations were measured in supernatants after 24 h of incubation as described in Example 6. Concentrations of different stimuli were included LPS, 0.5 μ g/ml; TNF- α , 20 ng/ml; IL-1 β , 50 ng/ml; and IFN- γ , 50 units/ml. Data were measured as the mean \pm S.D. of three different studies. Assay were conducted for control, LPS, LPS + TNF- α , LPS + IFN- γ , LPS + IL-1 β , LPS + TNF- α + forskolin, LPS + forskolin + IFN- γ , and LPS + forskolin + IL-1 β .

The decrease in LPS-induced iNOS expression with the increase in cAMP level and the increase in LPS-induced iNOS expression with the decrease in cAMP level clearly delineate cAMP and cAMP-dependent protein kinase as important regulators of iNOS biosynthesis in glial cells.

Table 8
Inhibition Of LPS-Induced Nitrite Accumulation In Rat Primary
Astrocytes By Different cAMP Agonists

Stimuli	Nitrite	Inhibition
	nmol/mg/24 h	%
Control	3.2 \pm 0.4	
LPS	31.4 \pm 3.6	0
LPS + forskolin	4.7 \pm 1.2	85
LPS + dideoxyforskolin	31.2 \pm 4.1	-
LPS + isoproterenol	8.1 \pm 2.3	74
LPS + IBMX	6.8 \pm 1.3	78
LPS + Rolipram	6.2 \pm 1.2	80

Primary astrocytes were cultured for 24 h in serum-free DMEM/F-12 with the listed reagents; nitrite concentration in the supernatants was then measured as described above. Concentration

of reagents were: LPS, 1.0 $\mu\text{g/ml}$; forskolin, 10 μM ; 1,9-dideoxyforskolin, 10 μM ; isoproterenol, 10 μM ; 1-isobutyl-1-methylxanthine (IBMX), mM; Rolipram, 10 μM . All the cAMP agonists were added to the cells 15 min prior to the addition of LPS. Data are mean \pm S.D. of three different studies.

5

Dose Dependence of Forskolin Inhibition of the LPS Stimulation of iNOS Astrocytes were incubated with different concentrations of forskolin 15 min before the addition of 1 $\mu\text{g/ml}$ LPS, and after 24 h the iNOS activity was measured as nitrite concentrations in the supernatant and conversion of arginine to citrulline in the cellular homogenates (FIG. 1). The level of nitrite and iNOS activity were inhibited to a similar degree at all the concentrations of forskolin tested. The lowest does of forskolin found to inhibit iNOS activity and NO production significantly (by 30%) was 0.1 μM . At 10 μM forskolin, NO production and iNOS activity were inhibited by about 90%. Higher doses of forskolin (50-100 μM) did not result in further significant inhibition of iNOS. This may be due to the fact that PKA was already completely activated in extracts of cells incubated with 10 μM forskolin. The PKA activity increased with the increase in forskolin concentration. The reciprocal relationship of production of NO and iNOS activity with PKA activity supports the conclusion that PKA plays a pivotal role in the regulation of iNOS expression in astrocytes.

Modulation of LPS-and/or Cytokine-mediated iNOS Expression by Compounds
Modulating Intracellular Levels of cAMP in Rat Primary Astrocytes Primary astrocytes were stimulated with TNF- α , IL-1 β , and IFN- γ alone or in different combinations for 24 h and iNOS was measured. TNF- α , IL-1 β , and IFN- γ individually were able to induce iNOS activity, protein, and mRNA, however, when tested in combinations between them or with LPS, the magnitude of induction was significantly higher. Cells incubated in serum-free DMEM/F-12 received 10 μM forskolin or 0.2 μM H-89 15 min before the addition of the different cytokines (TNF- α , 100 ng/ml; IL-1 β , 200 ng/ml; IFN- γ , 200 units/ml). Assay were conducted for control, TNF- α , IL-1 β , IFN- γ , TNF- α + H-89, IL-1 β + H-89, IFN- γ + H-89, TNF- α + forskolin, IL-1 β + forskolin, IFN- γ + forskolin. Activities for iNOS were measured in cell homogenates after 24 h as described. Results are expressed as means \pm S.D. of three different studies. Cell homogenates were immunoblotted with antibodies against mouse macrophage iNOS as described. After 6 h of incubation, cells were taken out from culture dishes directly by adding

Ultraspec-II RNA reagent (Biotech Laboratories Inc.) to isolate total RNA and northern blot analyses for iNOS mRNA were carried out as described. Forskolin, the activator of PKA, completely inhibited the cytokine-induced expression of iNOS, whereas H-89, a specific inhibitor of PKA, stimulated the cytokine-induced expression of iNOS.

5 Similarly, the induction of iNOS by several combinations of cytokines and LPS were also inhibited by forskolin in rat primary astrocytes, indicating that augmentation of the cellular levels of cAMP and the activation of cAMP-dependent protein kinase represents a general counter-regulatory mechanism for down-regulation of iNOS expression in astrocytes. Cells in this study were incubated in serum free DMEM/F-12 received 10 μ m forskolin (FOR) for 15
10 min before the addition of different combinations of LPS and cytokines. After 24 h of incubation, cell homogenates were analyzed for: iNOS activity, and iNOS protein by immunoblotting. After 6 h of incubation, cells were taken out and northern blot analyses for iNOS mRNA were carried out as described. Concentrations of different stimuli were: LPS, 0.5 μ g/ml; TNF- α , 20 ng/ml; IL-1 β , 50 ng/ml; IFN- γ , 50 units/ml. Assay were conducted for
15 control, TNF- α + IFN- γ , TNF- α + IL-1 β , LPS + IL-1 β , LPS + TNF- α , LPS + IFN- γ , TNF- α + IFN- γ + forskolin, TNF- α + IL-1 β + forskolin, LPS + IL-1 β + forskolin, and LPS + IFN- γ + forskolin.

EXAMPLE 4

20 **Therapy for X-Adrenoleukodystrophy: Normalization of very long chain fatty acids and inhibition of induction of cytokines by cAMP**

Materials and Methods

Reagents DMEM and bovine calf serum were from GIBCO. Forskolin, 1,9-dideoxyforskolin, 8-Br cAMP, S(p)-cAMP, H-89, rp-cAMP and rolipram were obtained
25 from Biomol, USA. C_{18:0}-CoA, NADPH and N-ethylmaleimide were from Sigma (USA). [2-¹⁴C]Malonyl-CoA and K¹⁴CN (52 mCi/mmol) were purchased from DuPont-New England Nuclear. [1-¹⁴C]Lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with K¹⁴CN as described previously (Hoshi and Kishimoto, 1973).

Enzyme assay for β -oxidation of lignoceric acid The enzyme activity of
30 [1-¹⁴C]lignoceric acid β -oxidation to acetate was measured in intact cells suspended in Hank's Buffered Salt Solution (HBSS). Briefly, the reaction mixture in 0.25 ml of HBSS contained

50-60 μg of protein and 6 μM $[1-^{14}\text{C}]$ lignoceric acid. Fatty acids were solubilized with α -cyclodextrin and β -oxidation of $[1-^{14}\text{C}]$ lignoceric acid was carried out as described previously (Singh *et al.*, 1984; Hashmi *et al.*, 1986; Lageweg *et al.*, 1991; Lazo *et al.*, 1988). The reaction was stopped after 1 h with 0.625 ml of 1 M KOH in methanol, and the denatured
5 protein was removed by centrifugation. The supernatant was incubated at 60°C for 1 h, neutralized with 0.125 ml of 6 N HCl, and partitioned with chloroform and methanol. Radioactivity in the upper phase is an index of $[1-^{14}\text{C}]$ lignoceric acid oxidized to acetate.

Transport of lignoceric acid into cultured skin fibroblasts Cells were incubated for 15 min at 37°C under isotonic conditions in HBSS with $[1-^{14}\text{C}]$ lignoceric acid (6 μM) solubilized
10 with α -cyclodextrin as described earlier (Singh *et al.*, 1984; Hashmi *et al.*, 1986; Lageweg *et al.*, 1991). Then cells were separated from the incubation medium by centrifugation through an organic layer of brominated hydrocarbons (Cornell, 1980). This was performed in micro tubes (1.5 ml) containing 50 μl of 0.25 M sucrose in HBSS (as cushion), an organic layer (400 μl) consisting of a mixture of bromododecane and bromodecane (7:4, v/v),
15 and an upper layer (500 μl) of cells in HBSS.

Protein kinase A assay Cell extracts were assayed for PKA activity as described (Graves *et al.*, 1993) and herein by measuring the phosphorylation of kemptide (0.17 mM) in the presence or absence of PKI peptide (15 μM). PKA activity was calculated as the amount of kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the
20 presence of PKI peptide.

Enzyme assay for fatty acid elongation The fatty acid elongation activity was assayed by the method of Tsuji *et al.* (Tsuji *et al.*, 1984). Briefly, the assay mixture contained 100 mM potassium phosphate (pH 7.2), 0.5 mM NADPH, 0.05 mM $[2-^{14}\text{C}]$ malonyl-CoA, 1 mM N-ethyl maleimide and 50-60 μg of protein in a total volume of 0.25 ml. The concentrations of
25 $\text{C}_{18:0}$ -CoA was 1 mM. The reaction was started at 37°C by the addition of total homogenate and stopped by the addition of 1.25 ml of 10% (w/v) KOH after 30 min incubation. After saponification at 100°C for 30 min, the solutions were acidified with 1 ml of 4N HCl and fatty acids were extracted with 2.5 ml of n-pentane three times. The radioactivities incorporated into fatty acids were measured with a liquid scintillation counter.

30 *Measurement of VLCFA in Fibroblasts* Fatty acid methyl ester (FAME) was prepared as described previously by Lepage and Roy (1986) with modifications. Fibroblast cells,

suspended in HBSS, were disrupted by sonication to form a homogeneous solution. An aliquot (200 μ l) of this solution was transferred to a glass tube and 5 g heptacosanoic (27:0) acid was added as internal standard and lipids were extracted by Folch partition. Fatty acids were transesterified with acetyl chloride (200 μ l) in the presence of methanol and benzene (4:1) for 2 h at 100°C. The solution was cooled down to room temperature followed by addition of 5 ml 6% potassium carbonate solution at ice-cooled temperature. Isolation and purification of FAME were carried out as detailed by Dacremont *et al.* (1995). Purified FAME, suspended in chloroform, were analyzed by gas chromatograph GC-15A attached with chromatopac C-R3A integrator from Shimadzu Corporation.

10 *Preparation of post-nuclear membrane and western blot analysis* The membranes were prepared as described previously (Contreras *et al.*, 1996). Briefly, the post-nuclear fraction was diluted with an ice-cold solution of 0.1 M sodium carbonate, 30 mM iodoacetamide, pH 11.5. After 30 min of incubation at 4°C, the membranes were sedimented by ultracentrifugation. The sedimented membranes were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with antibodies against ALDP as described (Contreras *et al.*, 1996).

15 *RNA isolation and Northern blot analysis* Cultured skin fibroblasts were taken out from culture flasks directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc.) and total RNA was isolated according to the manufacturer's protocol. Twenty micrograms of RNA from each sample were electrophoretically resolved on 1.2% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and cross-linked using UV Stratalinker (Stratagene, USA). Full length ALDP cDNA was obtained from Dr. Patrick Aubourg, INSERM, Hospital Saint-Vincent-de-Paul, Paris, France. ³²P-labeled cDNA probes were prepared according to the instructions provided with Ready-To-Go DNA labeling kit (Pharmacia Biotech). Northern blot analysis was performed essentially as described for Express Hyb Hybridization solution (Clontech) at 68°C. Actin cDNA probe was used as standard for comparing hybridization signals.

25 *Isolation of rat primary astrocytes and microglia* Astrocytes were prepared from rat cerebral tissue as described (McCarthy and De Vellis, 1980) and herein. Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (1986). For the induction of cytokine production, cells were stimulated with LPS in serum-free condition.

Determination of TNF- α and IL-1 β in culture supernatants Cells were stimulated with LPS in serum-free media for 24 h in the presence or absence of forskolin or rolipram, and concentrations of TNF- α and IL-1 β were measured in culture supernatants by using high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, USA) according to the manufacturer's instructions.

Results

Compounds that modulate the intracellular cAMP also modulate the β -oxidation of lignoceric acid and fatty acid chain elongation in X-ALD fibroblasts: First, the effect of cAMP derivatives on lignoceric acid β -oxidation in control human fibroblasts was examined. Cultured skin fibroblasts were treated with different activators and inhibitors of protein kinase A (PKA) and tested for β -oxidation of lignoceric acid. It is apparent from Table 9 that compounds known to increase cAMP (forskolin and 8-Br-cAMP) stimulated lignoceric acid β -oxidation whereas compounds known to decrease cAMP (H-89 and myristoylated PKI) inhibited lignoceric acid β -oxidation in control skin fibroblasts. The inactive analogue of forskolin, 1,9-dideoxyforskolin, was ineffective in stimulating β -oxidation (Table 9). These results indicate that PKA has a positive modulatory role on lignoceric acid β -oxidation. Since the β -oxidation of lignoceric acid is impaired in X-ALD patients, the inventor studied the effect of different activators and inhibitors of PKA on lignoceric acid β -oxidation in cultured skin fibroblasts of X-ALD. FIG. 2 shows that the compounds (forskolin, 8-bromo cAMP and rolipram) known to increase intracellular cAMP stimulated lignoceric acid β -oxidation (FIG. 2A) and activated the PKA activity (FIG. 2C). On the other hand, β -oxidation of lignoceric acid was inhibited by PKA inhibitors (H-89 and myristoylated PKI). A combination of forskolin (activator of PKA) and H-89 or myristoylated PKI (inhibitors of PKA) had relatively little effect on the activation of PKA as well as on the β -oxidation of lignoceric acid. These observations indicate that β -oxidation of lignoceric acid is modulated by cAMP and PKA.

However, in contrast to the effects on β -oxidation of lignoceric acid, activators of PKA inhibited the fatty acid chain elongation and inhibitors of PKA stimulated this activity in X-ALD fibroblasts (FIG. 2B). The increase in β -oxidation of lignoceric acid by forskolin and its inhibition by H-89 were dose-dependent. Cells in this experiment were incubated in serum-containing DMEM with different concentrations of forskolin (0-10 μ M) or H-89 (0-4 μ M) for 72 h. After every 24 h, media was replaced with the addition of fresh reagents.

β -oxidation of lignoceric acid (pmol/h/mg protein) was measured in cell-suspension as described in the methods section.

To understand the mechanism of cAMP-mediated stimulation of lignoceric acid β -oxidation, fibroblasts of X-ALD were treated with cAMP analogs, and the transport of
5 lignoceric acid into intact cells and β -oxidation of lignoceric acid in cell homogenates of X-ALD were measured. Similar to the modulation of lignoceric acid β -oxidation, activators of PKA also stimulated the transport of lignoceric acid into ALD cells by more than two fold whereas inhibitors of PKA inhibited the transport of lignoceric acid by 40 to 50 percent. Stimulation of lignoceric acid β -oxidation in cell homogenates of ALD fibroblasts as well as in
10 cell suspension (FIG. 2A) indicates that increase in β -oxidation is not due to an intracellular increase of substrate concentration but by stimulation of enzyme system for oxidation of lignoceric acid. In the cell, fatty acids are oxidized by mitochondrial and peroxisomal β -oxidation enzyme. Etomoxir, an inhibitor of mitochondrial β -oxidation of fatty acids (Mannaerts *et al.*, 1979), had no effect on cAMP-mediated stimulation of lignoceric acid
15 β -oxidation indicating that the observed stimulation of lignoceric acid was a peroxisomal function. The increase in β -oxidation and transport of lignoceric acid but the decrease in fatty acid chain elongation with the increase in cAMP level and PKA activity, and the decrease in β -oxidation and transport of lignoceric acid but the increase in fatty acid chain elongation with the decrease in cAMP level and PKA activity clearly delineate cAMP and cAMP-dependent
20 protein kinase A as important regulators of the metabolism of VLCFA.

Table 9
Effects of different agonists and antagonists of PKA on β -oxidation of lignoceric acid in control human fibroblasts

Treatments	Lignoceric acid β -oxidation (pmol/h/mg protein)
Control	565.2 \pm 48.3
Forskolin	885.3 \pm 62.1
1,9 dideoxy forskolin	571.4 \pm 39.6
8-Br-cAMP	872.0 \pm 53.7
H-89	405.6 \pm 44.1
Myristoylated PKI	432.3 \pm 46.5

Cells were treated for 72 h serum-containing DMEM with the listed reagents; β -oxidation of lignoceric acid was measured as described under "Material and Methods". Media was replaced after every 24 h with the addition of fresh reagents. Concentrations of reagents were: forskolin, 4 μ M; 1,9 dideoxy forskolin, 4 μ M; 8-Br-cAMP, 50 μ M; H-89, 1 μ M; myristoylated PKI, 0.2 μ M. Data are mean \pm S.D. of three different experiments.

Modulation of cellular content of VLCFA in X-ALD and AMN fibroblasts by compounds modulating intracellular levels of cAMP Since cAMP derivatives increase β -oxidation of lignoceric acid and decrease fatty acid chain elongation, the effect of cAMP derivatives on the level of VLCFA in X-ALD fibroblasts was examined. Treatment of X-ALD fibroblasts with 4 μ M of forskolin for different time periods (days) resulted in a time-dependent increase in oxidation of lignoceric acid and a time-dependent decrease $C_{22:0}$ in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ as shown in FIG. 3A-3C. Within 12 to 15 days of treatment, the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts decreased to the normal level. This decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ was also associated with the decrease in the absolute amounts of $C_{24:0}$ and $C_{26:0}$ whereas no significant change was observed in the levels of $C_{22:0}$ (behenic acid). To decipher the possible mechanism of this dramatic decrease of VLCFA, X-ALD fibroblasts were treated with different activators of PKA (forskolin, 8-Br-cAMP and rolipram) for 15 days and analyzed the level of VLCFA. The treatment of X-ALD cultured skin fibroblasts with compounds known to increase intracellular cAMP lowered the ratios of

$C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ to the normal level. Cells were incubated in serum-containing DMEM for 15 days with control, forskolin, 8-Br-cAMP, rolipram, forskolin + H-89, H-89, and IFN- β , and the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ were measured as described in Example 4. Concentrations of reagents were: forskolin, 4 μ M; 8-Br-cAMP, 50 μ M; rolipram, 10 μ M; H-89, 1 μ M; myristoylated PKI, 0.2 μ M; IFN- β , 50 U/ml. Data are mean \pm S.D. of three different experiments. The inactive forskolin analogue, 1,9-dideoxyforskolin, had no effect on the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$. However, compared to X-ALD fibroblasts, forskolin marginally lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in control skin fibroblasts. A two weeks treatment with forskolin lowered the ratio of $C_{26:0}/C_{22:0}$ from 0.04 to 0.029 and the ratio of $C_{24:0}/C_{22:0}$ from 1.23 to 1.12. Consistent with the effect of H-89 and myristoylated PKI on the β -oxidation of lignoceric acid, these two compounds blocked the observed effect of forskolin on the level of VLCFA when added along with forskolin indicating that cAMP analogs lower the level of VLCFA in X-ALD fibroblasts via activation of PKA. On the other hand, interferon- β , which has been indicated as a possible therapy for X-ALD based on favorable effects found in multiple sclerosis (Moser, 1995), was ineffective in lowering the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in skin fibroblasts of X-ALD.

Normalization of the levels of VLCFA by forskolin or rolipram in different X-ALD cells with or without deletion of the X-ALD gene Although the precise function of ALDP, an X-ALD gene product, in the metabolism of VLCFA is not known at the present time, accumulation of VLCFA in X-ALD cells with loss or mutations of ALDP and their normalization following transfection of cDNA for ALDP indicate a role of ALDP in the metabolism of VLCFA (Cartier *et al.*, 1995). Therefore, the inventor examined whether decrease in VLCFA in X-ALD fibroblasts by activators of PKA is mediated through the involvement of the ALD gene. ALDS1, ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene, whereas ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene. These cell lines were incubated in serum-containing DMEM with 4 μ M forskolin for 15 days or control medium, and the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$, β -oxidation of lignoceric acid and elongation of fatty acids were measured as described in Example 4. X-ALD cells with mutation or deletion of the ALD gene were treated with forskolin for two weeks and tested for the levels of ALDP protein and its mRNA, levels of VLCFA. Results were measured as the mean \pm S.D. of three different experiments.

It was apparent that treatment of X-ALD fibroblasts with forskolin for two weeks had no effect on the steady state levels of ALDP and its mRNA in X-ALD cells. However, forskolin normalized the level of VLCFA in X-ALD fibroblasts by decreasing the rate of fatty acid chain elongation and increasing the rate of β -oxidation of lignoceric acid despite the status of mRNA and protein of ALDP. Treatment of X-ALD fibroblasts with rolipram for two weeks also increased the oxidation of lignoceric acid between 50 to 65 percent and normalized the levels of VLCFA in these cell lines indicating that rolipram, an inhibitor of cAMP phosphodiesterase, has same effect on the metabolism of VLCFA in X-ALD cells with nonfunctional ALDP due to a mutation or with absence of ALDP due to a deletion of the X-ALD gene.

Forskolin and rolipram inhibit the induction of cytokine production in rat primary astrocytes and microglia Since both astrocytes and microglia, reactive glial cells, in the demyelinating lesions of ALD brain, are reported to express TNF- α and IL-1 β (Powers *et al.*, 1992; McGuinness *et al.*, 1995), the effect of cAMP derivatives on the induction of cytokine production in astrocytes and microglia was studied. Primary astrocytes in serum-free DMEM/F-12 were treated with different activators and inhibitors of PKA 15 min before the addition of 1 μ g/ml of lipopolysaccharide (LPS). FIG. 4 shows that the compounds (forskolin, 8-bromo-cAMP, and rolipram) known to increase intracellular cAMP inhibited the LPS-stimulated production of TNF- α (FIG. 4A) and IL-1 β (FIG. 4B), and activated PKA activity (FIG. 4C). On the other hand, LPS-stimulated production of TNF- α and IL-1 β were increased by inhibitors of PKA (H-89 and myristoylated PKI). The reciprocal relationship of induction of TNF- α and IL-1 β with PKA activity supports the conclusion that PKA plays a pivotal role in the regulation of proinflammatory cytokines in astrocytes. Similar to astrocytes, forskolin or rolipram also inhibited the LPS-induced production of TNF- α and IL-1 β , and H-89 stimulated the production of these proinflammatory cytokines in rat primary microglia (Table-10).

Table 10
Inhibition of LPS-induced production of TNF- α and IL-1 β in rat primary microglia
by forskolin and rolipram

Production of cytokines	Treatments		
	LPS only	LPS + Forskolin	LPS + Rolipram
TNF- α	14.1 \pm 2.1	0.9 \pm 0.1	1.2 \pm 0.09
IL-1 β	20.8 \pm 2.8	1.9 \pm 0.2	2.3 \pm 0.3

Cells preincubated with 10 μ M forskolin or 20 μ M of rolipram for 15 min in serum-free condition was stimulated with 1.0 μ g/ml of LPS. After 24 h of incubation, concentrations of TNF- α and IL- β were measured in supernatants as described in the methods section. TNF- α and IL- β are expressed as ng/24 h/mg protein. Data are expressed as the mean \pm S.D. of three different experiments.

EXAMPLE 5

Lovastatin and sodium phenylacetate normalize the level of very long chain fatty acids in skin fibroblasts of X-Adrenoleukodystrophy

10 Materials and Methods

Reagents DMEM, bovine calf serum and Hank's Buffered Salt Solution (HBSS) were from GIBCO. [1- 14 C]Lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with K 14 CN as described previously.

Enzyme assay for β -oxidation of lignoceric acid The enzyme activity of [1- 14 C]lignoceric acid β -oxidation to acetate was measured in intact cells suspended in HBSS. Briefly, the reaction mixture in 0.25 ml of HBSS contained 50-60 μ g of protein and 6 μ M [1- 14 C]lignoceric acid. Fatty acids were solubilized with α -cyclodextrin and β -oxidation of [1- 14 C]lignoceric acid was carried out as described previously (Singh *et al.*, 1984; Lazo *et al.*, 1988).

Measurement of VLCFA in Fibroblasts Fatty acid methyl ester (FAME) was prepared as described previously (Lepage and Roy, 1986) with modifications. Fibroblast cells, suspended in HBSS, were disrupted by sonication to form a homogeneous solution. An aliquot (200 μ l) of this solution was transferred to a glass tube and 5 μ g heptacosanoic (27:0) acid was

added as internal standard and lipids were extracted by Folch partition. Fatty acids were transesterified with acetyl chloride (200 μ l) in the presence of methanol and benzene (4:1) for 2 h at 100°C. The solution was cooled down to room temperature followed by addition of 5 ml 6% potassium carbonate solution at ice-cooled temperature. Isolation and purification of FAME were carried out as detailed by Dacremont *et al.* (1995). Purified FAME, suspended in chloroform, were analyzed by gas chromatograph GC-15A attached with chromatopac C-R3A integrator from Shimadzu Corporation.

Preparation of post-nuclear membrane and western blot analysis The membranes were prepared as described previously (Contreras *et al.*, 1996). Briefly, the post-nuclear fraction was diluted with an ice-cold solution of 0.1 M sodium carbonate, 30 mM iodoacetamide, pH 11.5. After 30 min of incubation at 4°C, the membranes were sedimented by ultracentrifugation. The sedimented membranes were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with antibodies against ALDP as described (Contreras *et al.*, 1996).

RNA isolation and Northern blot analysis Cultured skin fibroblasts were taken out from culture flasks directly by adding Ultraspec-II RNA reagent (Biotech Laboratories Inc.) and total RNA was isolated according to the manufacturer's protocol. Twenty micrograms of RNA from each sample were electrophoretically resolved on 1.2% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and cross-linked using UV Stratalinker (Stratagene, USA). Full length ALDP cDNA was obtained from Dr. Patrick Aubourg, INSERM, Hospital Saint-Vincent-de-Paul, Paris, France. ³²P-labeled cDNA probes were prepared according to the instructions provided with Ready-To-Go DNA labeling kit (Pharmacia Biotech). Northern blot analysis was performed essentially as described for Express Hyb Hybridization solution (Clontech) at 68°C. GAPDH cDNA probe was used as standard for comparing hybridization signals.

Results

Inhibitors of mevalonate pathway stimulate the β -oxidation of lignoceric acid in X-ALD fibroblasts First, the effect of mevalonate inhibitors (lovastatin, mevastatin and NaPA) on the β -oxidation of lignoceric acid in control human fibroblasts was examined. It is apparent from Table 11 that lovastatin, mevastatin and NaPA stimulated the β -oxidation of lignoceric acid in control human fibroblasts. Since the β -oxidation of lignoceric acid is impaired in X-ALD

patients, the effect of these compounds on lignoceric acid β -oxidation was studied in cultured skin fibroblasts of X-ALD.

Table 11
Lovastatin and NaPA stimulate the β -oxidation of lignoceric acid in control human skin fibroblasts

Treatments	Lignoceric acid β -oxidation (pmol/h/mg protein)
Control	570.2 \pm 52.3
Lovastatin (5 μ M)	945.7 \pm 105.6
Mevastatin (5 μ M)	889.6 \pm 78.4
NaPA (5 mM)	826.2 \pm 87.2

Cells were treated for 72 h in serum-containing DMEM with the listed reagents; β -oxidation of lignoceric acid was measured as described in the methods section. Media was replaced after every 24 h with the addition of fresh reagents. Data are mean \pm S.D. of three different studies.

Similar to control fibroblasts, these compounds also stimulated lignoceric acid β -oxidation in X-ALD skin fibroblasts. Cells were incubated in serum-containing DMEM with different concentrations of lovastatin (0-10 μ M) or NaPA (0-5 mM). After every 24 h, media was replaced with the addition of fresh reagents. Lignoceric acid β -oxidation was measured (pmol/h/mg protein) after 72 h in cell-suspension as described in the methods section. Values were determined as the mean \pm S.D. of three different studies. Both lovastatin and NaPA dose-dependently stimulated lignoceric acid β -oxidation in X-ALD fibroblasts. The highest dose of lovastatin found to stimulate lignoceric acid β -oxidation (by 70%) was 5 μ M whereas the highest dose of NaPA found to stimulate lignoceric acid β -oxidation (by 40%) was 5 mM. However, greater degree of stimulation (more than two fold) was observed by a combination of lovastatin and NaPA even at a dose lower than the one used individually. Higher doses of lovastatin (10-20 μ M) or NaPA (10-20 mM) were cytotoxic to the X-ALD fibroblasts and did not result in further significant stimulation. In the cell, fatty acids are oxidized by mitochondrial and peroxisomal β -oxidation enzyme. Etomoxir, an inhibitor of mitochondrial β -oxidation of fatty acids (Mannaerts *et al.*, 1979), had no effect on lovastatin- or

NaPA-mediated stimulation of lignoceric acid β -oxidation indicating that the observed stimulation of lignoceric acid β -oxidation was a peroxisomal function.

Modulation of cellular content of VLCFA in X-ALD fibroblasts by lovastatin and NaPA

Since mevalonate inhibitors increased β -oxidation of lignoceric acid in control as well as X-ALD fibroblasts, the inventor examined the effect of these compounds on the level of VLCFA in X-ALD fibroblasts. Treatment of X-ALD cultured skin fibroblasts with 5 μ M lovastatin for different time periods (days) resulted in a time-dependent decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$. Cells were incubated in serum-containing DMEM with 5 μ M lovastatin, 5 mM NaPA or the combination of 4 μ M lovastatin and 2 mM NaPA for 0-15 days, and the ratios of $C_{26:0}/C_{22:0}$ (24A) and $C_{24:0}/C_{22:0}$ (24B) were measured every 3 days as described in the methods section. Values are mean of two different studies. Within 12 to 15 days of treatment, the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts decreased to the normal level. Similar to lovastatin, NaPA also lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts almost to the normal level after 15 days of treatment. However, consistent with the higher degree of stimulation of lignoceric acid β -oxidation by a combination of lovastatin and NaPA, the same combination lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ to the normal level within 7 days. This decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ was also associated with the decrease in the absolute amounts of $C_{24:0}$ and $C_{26:0}$ whereas no significant change was observed in the levels of $C_{22:0}$ (behenic acid).

Normalization of the levels of VLCFA by lovastatin or NaPA in different X-ALD cells with or without deletion of the X-ALD gene Although the precise function of ALDP, X-ALD gene product, in the metabolism of VLCFA is not known at the present time, however, accumulation of VLCFA in X-ALD cells with loss or mutations of ALDP and their normalization following transfection of cDNA for ALDP indicate a role of ALDP in the metabolism of VLCFA (Cartier *et al.*, 1995). Therefore, the inventor examined whether lovastatin or NaPA were able to lower the level of VLCFA in different X-ALD fibroblasts with mutation or deletion of the X-ALD gene. The status of ALDP mRNA or protein and the rate of β -oxidation of lignoceric acid (Table 12) in different X-ALD fibroblasts indicates that ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene. It is apparent from Table 3 that treatment of X-ALD fibroblasts with lovastatin or NaPA or the

combination of these two stimulated the β -oxidation of lignoceric acid (55-80%) and normalized the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ indicating that these drugs are capable of lowering the level of VLCFA in X-ALD fibroblasts to the normal level, irrespective of mutation or deletion of the X-ALD gene, the candidate gene for X-ALD.

5

Table 12-A

Effect of lovastatin and NaPA on (A) β -oxidation of lignoceric acid and (B) the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in cultured skin fibroblasts of X-ALD

	Lignoceric acid β -oxidation (pmol/h/mg protein)			
	Control	Lovastatin	NaPA	Lovastatin + NaPA
ALDS2	142.7 \pm 15.7	223.5 \pm 24.1	202.5 \pm 17.4	274.6 \pm 30.5
ALDS5	154.2 \pm 14.2	248.2 \pm 26.2	211.5 \pm 22.6	296.2 \pm 25.6
ALDS6	132.4 \pm 15.9	218.3 \pm 19.8	189.7 \pm 21.2	250.1 \pm 28.3
ALDS3	122.3 \pm 11.7	201.3 \pm 22.3	183.2 \pm 17.3	248.6 \pm 29.6
ALDS4	118.5 \pm 12.6	192.8 \pm 20.5	178.9 \pm 18.3	238.7 \pm 21.1

Table 12-B
Effect of lovastatin and NaPA on (A) β -oxidation of lignoceric acid and (B) the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in cultured skin fibroblasts of X-ALD

Cell Lines	$C_{26:0}/C_{22:0}$			$C_{24:0}/C_{22:0}$		
	Control	Lovastatin	Lovastatin +NaPA	Control	Lovastatin	Lovastatin +NaPA
ALDS2	0.17±0.022	0.049 ± 0.01	0.04 ± 0.008	1.84±0.25	1.25 ± 0.15	1.14 ± 0.15
ALDS5	0.18±0.025	0.055±0.008	0.04 ± 0.007	1.94± 0.29	1.28 ± 0.21	1.18 ± 0.12
ALDS6	0.22±0.034	0.058 ± 0.01	0.045± 0.008	2.01 ± 0.3	1.31 ± 0.18	1.21 ± 0.14
ALDS3	0.16±0.024	0.045 ± 0.06	0.03 ± 0.005	1.88± 0.21	1.26 ± 0.16	1.19 ± 0.25
ALDS4	0.19±0.028	0.052 ± 0.07	0.036± 0.006	1.96± 0.23	1.29 ± 0.02	1.22 ± 0.15

Cells were incubated in serum-containing DMEM with 5 μ M lovastatin, 5 mM NaPA or the combination of 4 μ M forskolin and 2 mM NaPA for 15 days, and the β -oxidation of lignoceric acid (A) and the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ (B) were measured as described in the methods section. Results are mean \pm S.D. of three different studies. ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene.

The results of the preceding Examples 1-4 that lovastatin and NaPA inhibit the induction of nitric oxide synthase and proinflammatory cytokines (TNF- α , IL-1 β and IL-6) in rat primary astrocytes, microglia and macrophages indicates that these drugs, alone or in combination, represent a novel approach for therapeutics directed against cytokine- and NO-mediated brain disorders, particularly in demyelinating conditions. Lovastatin and NaPA have already been approved for medication/drug trials on human diseases. Therefore, normalization of VLCFA by lovastatin and NaPA in X-ALD fibroblasts indicates that these drugs may be used to lower the level of VLCFA and ameliorate the myelinolytic inflammation in X-ALD patients.

EXAMPLE 6

Inhibitors of Phosphatase 1 and 2A Differentially Regulate Expression of iNOS

Materials and Methods

Reagents Recombinant rat IFN- γ , DMEM/F-12 medium, fetal bovine serum, Hanks' balanced salt solution (HBSS) and NF- κ B DNA binding protein detection kit were from GIBCO. Human IL-1 β was from Genzyme. Mouse recombinant TNF- α was obtained from Boehringer Mannheim, Germany. LPS (*Escherichia coli*) was from Sigma. N^G-methyl-L-arginine (L-NMA), okadaic acid, calyculin A, cantharidin and antibodies against mouse macrophage iNOS were obtained from Calbiochem, USA. Deltamethrin and fenvalerate were obtained from Biomol, USA. [γ -³²P]ATP (3000 Ci/mmol) were from Amersham, USA.

Induction of NO production in astrocytes and C₆ glial cells Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (McCarthy and DeVellis, 1980). Cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (FBS).

After 10 days of culture astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. The shaking was repeated two more times after a gap of one or two days time before subculturing to ensure the complete removal of all the oligodendrocytes and microglia. Cells were trypsinized, subcultured and stimulated with LPS or different cytokines in serum-free DMEM/F-12. C₆ glial cells obtained from ATCC was also maintained and induced with different stimuli as above.

Isolation of rat macrophages and induction of NO production Resident macrophages were obtained from rat by peritoneal lavage with sterile RPMI 1640 medium containing 1% fetal bovine serum and 100 µg/ml gentamicin as described herein. Cells were washed three times with RPMI 1640 at 4°C. All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO₂ in air. Macrophages at a concentration of 2×10^6 /ml in RPMI 1640 medium containing L-glutamine and gentamicin were added in volumes of 800 µl to a 35 mm plate. After 1 h, nonadherent cells were removed by washing and 800 µl of serum-free RPMI 1640 medium with various stimuli were added to the adherent cells. After incubation in 5% CO₂ in air at 37°C, culture supernatants were transferred to measure NO production.

Assay for NO synthesis Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 400 µl of culture supernatant was allowed to react with 200 µl of Griess reagent and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all studies. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

In vitro PP1/2A assay The extraction and assay for PP1/2A were performed as described (Begum and Ragolia, 1996). Control and treated cells were scraped off the dishes with 0.3 ml of phosphatase extraction buffer containing 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 10 µg/ml each of aprotinin, leupeptin, antipain, soybean trypsin inhibitor, 1 mM benzamide, and 1 mM PMSF. The cells were sonicated for 10 s and centrifuged at $2000 \times g$ for 5 min, and the supernatants were used for the assay of phosphatase activities using the protein phosphatase assay kit (Life Technologies, Inc.) according to the manufacturer's protocol.

Immunoblot analysis for iNOS Following 24 h incubation in the presence or absence of different stimuli, cells were scraped off, washed with Hank's buffer, and homogenized in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors. After electrophoresis the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and [¹²⁵I]-labeled protein A.

RNA isolation and Northern blot analysis Cells were taken out from culture dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc.) and total RNA was

isolated according to the manufacturer's protocol. For Northern blot analyses, 20 µg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, electrotransferred to Hybond-Nylon Membrane (Amersham) and hybridized at 68°C with ³²P-labeled cDNA probe using Express Hyb hybridization solution (Clontech) as described by the manufacturer. The cDNA probe was made by PCRTM amplification using two primers (forward primer: 5'-CTCCTTCAAAGAGGCAAAAATA-3' (SEQ ID NO:1); reverse primer: 5'-CACTTCCTCCAGGATGTTGT-3' (SEQ ID NO:2) (Geller *et al.*, 1993). After hybridization filters were washed two or three times in solution I (2X SSC, 0.05% SDS) for one hour at room temperature followed by solution II (0.1X SSC, 0.1 % SDS) at 50°C for another hour. The membranes were then dried and exposed with X-ray films (Kodak). The same filters were stripped and rehybridized with probes for GAPDH. The relative mRNA content for iNOS was measured after scanning the bands with a Biorad (Model GS-670) imaging densitometer.

Preparation of nuclear extracts and electrophoretic mobility shift assay Nuclear extracts from stimulated or unstimulated astrocytes (1 × 10⁷ cells) were prepared as described (Dignam *et al.*, 1983) with slight modification. Cells were harvested, washed twice with ice-cold phosphate-buffered saline and lysed in 400 µl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin) containing 0.1% Nonidet P40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 µl of buffer B (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 µl of modified buffer C (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and stored at -70°C until use. Nuclear extracts were used for the electrophoretic mobility shift assay using the NF-kB DNA binding protein detection system kit (GIBCO/BRL), according to the manufacturer's protocol.

Construction of reporter plasmid, transfection and assay of chloramphenicol acetyl transferase activity The CAT (chloramphenicol acetyl transferase) under the control of nitric oxide synthase promoter (iNOS) was created by subcloning 1.5 kb promoter from pGEM-NOS at *Sph* I and *Sal* I restriction sites of pCAT-basic vector (Promega). Full length promoter

(Eberhardt *et al.*, 1996) was amplified by using two primers (Forward: 5'-GAGAGTGTGCAAGTATTTGTAGGAG-3' (SEQ ID NO:6) and reverse: 5'-AAGGTGGCTGAGAAGTTTCA-3' (SEQ ID NO:7)) from rat genomic DNA and cloned in pGEM-T vector (Promega) to produce pGEM-NOS. The clone was confirmed by restriction mapping and sequencing. The cells were transfected by using the lipofectin (Life Technologies Inc., USA) method, as has been described in manufacturer's protocol, with 2 µg of reporter plasmid. They were then stimulated 24 h after transfection and harvested after 14 h of stimulation. CAT activity was measured as has been described.

Cell viability: Cytotoxic effects of all the inhibitors were determined by the MTT assay measuring the metabolic activity of cells.

Results

Inhibitors of PP1/2A stimulate LPS-induced production of NO in rat primary astrocytes: Rat primary astrocytes were cultured in serum-free DMEM/F-12 in the presence of LPS and inhibitors of different protein phosphatases. The concentration of NO as nitrite (a stable reaction product of NO with molecular oxygen) was measured in culture supernatants after 24 h. It is evident from Table 13 that bacterial LPS at a concentration of 1.0 µg/ml induced the production of NO as nitrite by about 8 fold. L-NMA, a competitive inhibitor of NOS suppressed LPS-mediated nitrite secretion indicating that LPS-induced nitrite release in rat primary astrocytes is dependent on NOS-mediated arginine metabolism (Table 13). Inhibitors of protein phosphatase (PP) 1/2A (calyculin A and microcystin), PP 2B (deltamethrin and fenvalerate) or protein tyrosine phosphatase (dephostin and orthovanadate) alone was neither stimulatory nor inhibitory to nitrite production in control astrocytes. However, calyculin A and microcystin, when added with the addition of LPS, potentially stimulated LPS-mediated induction of nitrite production in astrocytes. In contrast, inhibitors of PP 2B (cypermethrin, deltamethrin and fenvalerate) had no effect on LPS-induced nitrite production in astrocytes indicating that stimulation of LPS-induced production of NO in astrocytes is specific for the inhibitors of PP 1/2A.

To understand the mechanism of stimulatory effect of inhibitors of PP 1/2A on the LPS-mediated nitrite production in astrocytes, the effect of these inhibitors on the protein and mRNA level of inducible nitric oxide synthase (iNOS) was examined. Rat primary astrocytes were incubated in serum-free DMEM/F-12 received calyculin A, microcystin or cantharidin

along with 1.0 µg/ml of LPS. After 24 h, concentration of nitrite was measured in the supernatants as described in Example 6. Data were taken as the mean ± S.D. of three different experiments. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described in Example 6. After 6 h of incubation, cells were taken out directly by adding ultraspec-II RNA reagent (Biotecx Laboratories Inc) to the plates for isolation of total RNA, and northern blot analysis for iNOS mRNA was carried out as described. Assays were conducted for control, LPS, LPS + calyculin A (1 nM), LPS + calyculin A (2 nM), LPS + microcystin (1 nM), LPS + microcystin (2 nM), LPS + cantharidin (200 nM), and LPS + cantharidin (400 nM). Consistent with the production of nitrite, western blot analysis with antibodies against murine macrophage iNOS and northern blot analysis for iNOS mRNA of LPS-stimulated astrocytes clearly showed that inhibitors of PP 1/2A (calyculin A, microcystin and cantharidin) enhanced the LPS-mediated induction of iNOS protein and mRNA.

Since the inhibitors of PP 1/2A stimulated the LPS-mediated induction of iNOS, the inventor examined whether these inhibitors inhibited the activities of PP 1/2A in LPS-treated astrocytes. The activities of PP 1/2A were measured in homogenates after 30 min of incubation. Cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid (0-20 nM) along with 1.0 µg/ml of LPS. After 30 min of incubation, protein phosphatase activity was measured (nmol PI/mln/mg). Data was measured as the mean ± S.D. of three different experiments. Cells incubated in serum free DMEM/F-12 received different concentrations of okadaic acid in the presence or absence of 1.0 µg/ml of LPS. After 24 h of incubation, nitrite concentrations (nmol/mg/24 h) were measured in supernatants. Data are mean ± S.D. of three different experiments. Okadaic acid dose-dependently inhibited the activities of PP 1/2A and stimulated the LPS-mediated induction of iNOS protein and production of NO in astrocytes. In a similar manner, calyculin A also inhibited the activities of PP 1/2A.

Cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid (0, 1, 2, 4, 8, 15, and 20 nM) along with 1.0 µg/ml of LPS. After 24 h of incubation, cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described before.

Table 13
Effect of inhibitors of different protein phosphatases on LPS-induced
production of NO in rat primary astrocytes

Stimuli	Nitrite (nmol/rag/24 h)
Control	3.1 ± 0.3
LPS	28.2 ± 3.1
LPS + L-NMA (0.1 mM)	5.2 ± 0.4
LPS + Cypermethrin (1 nM)	27.6 ± 2.7
LPS + Deltamethrin (1 nM)	26.8 ± 2.9
LPS + Fenvalerate (20 nM)	27.1 ± 2.1
LPS + Calyculin A (2 nM)	67.8 ± 7.3
LPS + Microcystin (2 nM)	64.8 ± 7.2

Astrocytes preincubated in serum-free DMEM/F-12 for 30 min with L-NMA and different inhibitors of protein phosphatases received LPS (1.0 µg/ml). After 24 h of incubation, nitrite concentration in the supernatants were measured as described under "Materials and Methods". Data are expressed as the mean ± S.D. of three different experiments.

5

Stimulation of LPS- and cytokine-induced production of NO by calyculin A in C₆ glial cell Similar to primary astrocytes, proinflammatory cytokines and LPS induce the production of nitrite as well as the expression of iNOS in rat C₆ glial cells (Feinstein *et al.*, 1994a; Dobashi *et al.*, 1997). Unlike astrocytes, neither LPS or cytokine(s) alone was not a sufficient inducer of NO production in rat C₆ glial cells (Feinstein *et al.*, 1994a; Dobashi *et al.*, 1997). A combination of LPS and cytokines was required to induce the production of NO in C₆ glial cells (Feinstein *et al.*, 1994a; Dobashi *et al.*, 1997).

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However, the addition of 2 nM calyculin A along with LPS and cytokines to C₆ cells stimulated the expression of iNOS protein and the production of NO (nmol/mg/24 hr) by more than three fold in C₆ glial cells. Samples tested included control, LPS + TNF-α, TNF-α + IFN-γ, TNF-α + IL-1β, LPS + TNF-α + calyculin A, TNF-α + IFN-γ + calyculin A, and TNF-α + IL-1β + calyculin A. After 24 h, concentration of nitrite was measured in the supernatants as described. Data was measured as the mean ± S.D. of three different experiments. Cells

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incubated in serum-free DMEM/F-12 received calyculin A along with LPS and cytokines. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described. These results indicate that both in primary astrocytes and C6 glial cells the inhibitors of PP 1/2A up regulate the cytokine-induced expression of iNOS and the production of NO.

Inhibition of LPS- and cytokine-induced NO production by inhibitors of PP1/2A in rat peritoneal macrophages: Since inhibitors of PP 1/2A stimulated the LPS- and cytokine-induced NO production in rat primary astrocytes and C6 glial cells, the effect of these inhibitors on NO production and expression of iNOS in rat resident macrophages was examined. Similar to astrocytes, inhibitors of PP 1/2A alone had no effect on the induction of NO production. However, in contrast to the stimulation of NO production in astrocytes (Table 13), all three inhibitors of PP1/2A (calyculin A, microcystin and cantharidin) inhibited the LPS-induced NO production in rat peritoneal macrophages. Cells in this study were incubated in serum-free DMEM/F-12 and received calyculin A, microcystin or cantharidin along with 1.0 µg/ml of LPS. Samples tested included control, LPS, LPS + calyculin A (1 nM), LPS + calyculin A (2 nM), LPS + microcystin (1 nM), LPS + microcystin (2 nM), LPS + cantharidin (200 nM), and LPS + cantharidin (400 nM). After 24 h, concentration of nitrite was measured (nmol/mg/24 h) in the supernatants as described in Example 6. Data was measured as the mean ± S.D. of three different experiments. Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described. After 6 h of incubation, cells were analyzed for iNOS mRNA by northern blotting technique as described earlier. This decrease in NO production was accompanied by a decrease in iNOS protein and iNOS mRNA.

Okadaic acid, another very specific and potent inhibitor of PP 1/2A, also dose-dependently inhibited the LPS-mediated production of NO and expression of iNOS protein in astrocytes. Cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid (0, 5, 10, 15, and 20 nM in the presence or absence of 1.0 µg/ml of LPS. After 24 h of incubation, nitrite concentrations were measured in supernatants. Data was determined as the mean ± SD. of three different studies. Cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid along with 1.0 µg/ml of LPS. Samples tested included control, LPS, LPS + okadaic acid (1 nM), LPS + okadaic acid (2 nM),

LPS + okadic acid (4 nM), LPS + okadic acid (8 nM), LPS + okadic acid (15 nM), LPS + okadic acid (20 nM). After 24 h of incubation, cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as described in Example 6.

5 Similar to macrophages, calyculin A was also found to inhibit the LPS- and cytokine-induced production of NO and the expression of iNOS protein in the murine macrophage cell line RAW 264.7. Cells in this study were incubated in serum-free DMEM/F-12 received calyculin A along with LPS and cytokines. Samples tested included control, LPS + TNF- α , TNF- α + IFN- γ , TNF- α + IL-1 β , LPS + TNF- α + calyculin A, TNF- α
10 + IFN- γ + calyculin A, and TNF- α + IL-1 β + calyculin A. After 24 h, concentration of nitrite (nmol/mg/ 24 h) was measured in the supernatants as described in Example 6. Data are mean \pm S.D. of three different studies. Cell homogenates were electrophoresed, transferred on antibodies against mouse macrophage iNOS as described. Taken together, these results indicate that PP 1/2A activities are required to induce iNOS gene expression in macrophages.

15 *Differential effect of okadaic acid on iNOS promoter-derived chloramphenicol acetyl transferase (CAT) activity in LPS-stimulated rat primary astrocytes and macrophages*
Differential regulation of the induction of iNOS mRNA and protein in astrocytes and macrophages by the inhibitors of PP 1/2A indicates that these inhibitors regulate the transcription of iNOS gene differentially in these two different cell lines. Therefore, to
20 understand the effect of okadaic acid on the transcription of iNOS gene, astrocytes and macrophages were transfected with a construct containing the iNOS promoter fused to the CAT gene, and activation of this promoter was measured after stimulating the cells with LPS in the presence or absence of okadaic acid. Consistent with the effect of okadaic acid on the production of NO and the expression of iNOS in two different cell types, okadaic acid
25 stimulated the LPS-induced CAT activity in astrocytes but inhibited the LPS-induced CAT activity in macrophages (FIG. 5) indicating that okadaic acid differentially regulates the transcription of iNOS gene in astrocytes and macrophages.

Effect of okadaic acid on the activation of NF- κ B in rat primary astrocytes and macrophages
Inhibitors of PP 1/2A stimulated the induction of iNOS in astrocytes but
30 inhibited the induction of iNOS in macrophages indicating that PP 1/2A transduce different signals in two different cell types for the differential regulation of iNOS. Since the activation

of NF-kB is necessary for the induction of iNOS, to understand the basis of this differential regulation of induction of iNOS by inhibitors of PP 1/2A, the effect of okadaic acid on the LPS-induced activation of NF-kB in astrocytes and macrophages was examined. Astrocytes and macrophages incubated in serum-free DMEM/F-12 were treated with okadaic acid alone or together with LPS (1.0 µg/ml), and nuclear proteins were isolated. After 1 h of incubation, cells were taken out to prepare nuclear extracts and nuclear proteins were used for the electrophoretic mobility shift assay as described in Example 6. Samples assayed included nuclear extract of control cells, nuclear extract of LPS-treated cells, nuclear extract of LPS-treated cells incubated with 100-fold excess of unlabelled oligonucleotide, nuclear extract of cells treated with okadaic acid (5 nM) alone, nuclear extract of cells treated with okadaic acid (10 nM) alone, nuclear extract of LPS and okadaic acid (5 nM) treated cells, and nuclear extract of LPS and okadaic acid (10 nM) treated cells. Activation of NF-kB was evaluated by the formation of a distinct and specific complex in a gel-shift DNA-binding assay. Treatment of astrocytes or macrophages with 1.0 µg/ml of LPS resulted in the activation of NF-kB.

This gel shift assay detected a specific band in response to LPS that was competed off by an unlabelled probe. Although okadaic acid alone at different concentrations failed to induce the activation NF-kB in astrocytes yet okadaic acid alone induced the activation of NF-kB in macrophages. However, in both astrocytes and macrophages, okadaic acid stimulated the LPS-induced activation of NF-kB.

Inhibitors of PP 1/2A stimulate the LPS-induced production of TNF-α in rat primary astrocytes and macrophages: Okadaic acid stimulated the transcription of iNOS in astrocytes and attenuated the transcription of iNOS in macrophages. However, in contrast, okadaic acid stimulated the activation of NF-kB in both astrocytes and macrophages. Since the induction of TNF-α also depends on the activation of NF-kB, the effect of okadaic acid on the LPS-induced production of TNF-α in astrocytes and macrophages was studied. Consistent with the stimulatory effect of okadaic acid on the LPS-induced activation of NF-kB, okadaic acid stimulated the LPS-induced production of TNF-α in both astrocytes and macrophages (Table 14).

Table 14

Effect of inhibitors of PP 1 and PP 2A on LPS-induced production of TNF- α in rat primary astrocytes and macrophages

Stimuli	TNF- α (ng/24 h/mg protein)	
	Astrocytes	Macrophages
Control	0.3 \pm 0.03	0.5 \pm 0.06
LPS	5.8 \pm 0.7	18.9 \pm 5.2.3
LPS + Calyculin A (1 nM)	12.5 \pm 1.6	27.5 \pm 3.1
LPS+CalyculinA(2nM)	16.9 \pm 2.1	31.2 \pm 3.6
LPS + Okadaic acid (5 nM)	10.8 \pm 1.2	24.3 \pm 1.9
LPS + Okadaic acid (10 nM)	14.6 \pm 1.8	28.9 \pm 3.4

Cells preincubated in serum-free DMEM/F-12 with different concentrations of okadaic acid for 30 min was stimulated with 1.0 μ g/ml of LPS. After 24 h of incubation, concentration of TNF- α was measured in supernatants as described under "Materials and Methods". Data are expressed as the mean \pm S.D. of three different experiments.

Effect of inhibitors of PP1/2A on cell viability Astrocytes or macrophages were incubated with different inhibitors of PP1/2A for 24 h and their viability was determined as measured by the MTT assay. None of the inhibitors at the concentrations used in this study decreased or increased the viability of the cells. Therefore, stimulation of the expression of iNOS in astrocytes and inhibition of the expression of iNOS in macrophages by inhibitors of PP 1/2A are not due to any change in viability of either astrocytes or macrophages.

EXAMPLE 7

Cytokine-Mediated Induction Of Ceramide Production Is Redox-Sensitive

Materials and Methods

Reagents DMEM/F-12 and fetal bovine serum (FBS) were from GIBCO. Human IL1 β was from Genzyme. Mouse recombinant TNF- α was obtained from Boehringer Mannheim, Germany. Diamide, buthione (S,R)-sulfoximine, N-acetyl cysteine, pyrrolidine dithiocarbamate were from Sigma.

Isolation and maintenance of rat primary microglia, oligodendrocytes and astrocytes

Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (1986). Briefly, after 7 days the mixed glial cultures were washed 3 times with DMEM/F-12 containing 10% FBS and subjected to a shake at 240 rpm for 4 h at 37°C on a rotary shaker. The floating cells were washed and seeded onto plastic tissue culture flasks and incubated at 37°C. After 30 min the non-attached cells (mostly oligodendrocytes) were removed by repeated washes and the attached cells were used as microglia. These cells were seeded onto new plates for further studies. Ninety to ninety-five percent of this preparation was positive for nonspecific esterase, a marker for macrophages and microglia.

After 4 h shaking, the flasks were washed three times to remove the floating cells. Medium with 10% FBS was added and flasks were subjected to another cycle of shaking for 24 h at 250 rpm. The suspended cells were spun at 200 g and incubated for 30 min in tissue culture dish. The non-attached or weakly attached cells (mostly oligodendrocytes) were removed and seeded onto polylysine coated dishes and cultured in medium containing 1% FBS. Ninety-five to ninety-seven percent of these cells were positive for galactocerebroside immunostaining.

Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (1980). After 10 days of culture astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the complete removal of all oligodendrocytes and microglia, the shaking was repeated twice after a gap of one or two days. Attached cells were trypsinized (1 mM EDTA and 0.05% trypsin in 10 mM tris-buffer saline, pH 7.4) and distributed into culture dishes. These cells when checked for the astrocyte marker glial fibrillar acidic protein (GFAP), were found to be 95 to 100% positive. C6 glial cells obtained from ATCC were also maintained in DMEM/F-12 containing 10% FBS as indicated above.

Brain tissue Frozen and fixed X-adrenoleukodystrophy and multiple sclerosis brain tissues were obtained from Brain and Tissue Banks for Developmental Disorders, University of Maryland, Baltimore, MD 21201.

Lipid extraction Approximately 1.0×10^6 cells were exposed to different cytokines in the presence or absence of antioxidants for different periods and lipids were extracted according to the methods described by Welsh (1996).

Quantification of sphingomyelin by HPTLC and densitometry Sphingomyelin was separated from total lipid extracts by HPTLC (LPK-plates from Whatman Labsales, USA) as described (Ganser *et al.*, 1988) for phospholipids with the modification, that the plate was overrun for 30 min during its development and was dried overnight in vacuum desiccator.

5 Sphingomyelin was quantitated by densitometric scanning using Imaging Densitometer (Model GS-670; Bio-Rad, USA) and software provided with the instrument by the manufacturer.

Quantification of ceramide levels by diacylglycerol kinase assay Ceramide content was quantified essentially according to Priess *et al.* using diacylglycerol (DAG) kinase and [γ - 32 P]ATP (Priess *et al.*, 1986). Briefly, dried lipids were solubilized in 20 μ l of an octyl

10 β -D-glucoside/cardiophilin solution (7.5% octyl β -D-glucoside, 5 mM cardiophilin in 1 mM DTPA) by sonication in a sonicator bath. The reaction was then carried out in a final volume of 100 μ l containing the 20 μ l sample solution, 50 mM imidazole HCl, pH 6.6, 50 mM NaCl, 12.5 mM MgCl_2 , 1 mM EGTA, 2 mM dithiothreitol, 6.6 μ g of DAG kinase, and 1 mM [γ - 32 P]ATP (specific activity of 1.5×10^5 cpm/nmol) for 30 min at room temperature. The labeled

15 ceramide 1-phosphate was resolved with a solvent system consisting of methyl acetate: n-propanol: chloroform: methanol: 0.25% KCl in water: acetic acid (100:100:100:40:36:2). A standard sample of ceramide was phosphorylated under identical conditions and developed in parallel. Both standard and samples had identical R_f value (0.46). Quantification of ceramide 1-phosphate was carried out by autoradiography and densitometric scanning using Imaging

20 Densitometer (Model GS-670; Bio-Rad, USA). Values are expressed either as arbitrary units (absorbance) or as percent change.

Measurement of reduced glutathione (GSH) concentration in rat primary astrocytes Concentration of intracellular GSH was measured using a colorimetric assay kit for GSH from RandD, USA. Briefly, 2×10^6 cells were homogenized in 500 μ l of ice-cold 5%

25 metaphosphoric acid and centrifuged at 3000 g for 10 min. Supernatants were used to assay GSH using 4-chloro-l-methyl-7-trifluoromethyl-quinolinium methylsulfate and 30% NaOH at 400 nm.

Detection of DNA fragmentation Cells (1×10^6) were pelleted in an eppendorf tube by centrifugation at 1,000 rpm for 5 min, washed with PBS (pH 7.4), resuspended gently in 50 μ l

30 of a lysis buffer [200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 40 mM EDTA (pH 8.0), 0.5% SDS, 400 ng RNase A/ μ l] and incubated at 37°C for 1 h. The lysate received 200 μ l of the

digestion buffer [200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% SDS, 125 ng proteinase K/ μ l]. The contents were mixed by inversion several times and then incubated at 50°C for 2 h. An equal volume of a mixture phenol (pH 8.0), chloroform and isoamyl alcohol (25:24:1, v/v) was added, gently mixed for 10 min, and stored at room temperature for 2 min. The two phases
5 were separated by centrifugation at 3,000 rpm for 10 min. The viscous aqueous phase was transferred to a fresh tube and the phenol/chloroform extraction was repeated. The aqueous phase was extracted with an equal volume of chloroform and 1.0 M $MgCl_2$ was added to the aqueous phase to a final concentration of 10 mM. The total DNA was precipitated by the addition of 2 vols. of absolute ethanol with several inversions. DNA was pelleted by
10 centrifugation at 3,000 rpm for 15 min, washed with 70% ethanol and air-dried. The pellet was dissolved in 25 μ l of 10 mM Tris-HCl containing 1.0 mM EDTA (pH 8.0) and electrophoresed in 1.8% agarose gel at 4°C. The gel was stained with ethidium bromide and DNA-intercalated ethidium fluorescence was photographed on Polaroid film 665 (P/N) using an orange filter. To study DNA fragmentation in banked human brain tissues, brain tissues were gently
15 homogenized in 0.85 M sucrose buffer and nuclei were purified according to the procedure described previously (Lazo *et al.*, 1991). Total genomic DNA was isolated from the nuclei and electrophoresed as described.

Fragment end labeling of DNA on paraffin-embedded tissue sections of MS and X-AID brains Fragmented DNA was detected in situ by the terminal deoxynucleotidyl transferase
20 (TdT)-mediated binding of 3'-OH ends of DNA fragments generated in response to apoptotic signals, using a commercially available kit (TdT FragEL™) from Calbiochem, USA. Briefly, paraffin-embedded tissue slides were deparaffinized, rehydrated in graded ethanol, treated with 20 μ g/ml proteinase K for 15 min at room temperature, and washed prior to TdT staining. After TdT staining, sections were lightly counterstained with methyl green.

25 Results

N-Acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) block TNF- α - and IL-1 β -induced degradation of sphingomyelin to ceramide in primary rat astrocytes Rat primary astrocytes were cultured in serum-free media with TNF- α or IL-1 β for different times to quantify the production of ceramide using diacylglycerol (DAG) kinase. Since DAG kinase
30 phosphorylates both DAG and ceramide using [γ -³²P]ATP as substrate, both lipids can be quantified in the same assay. Cells were exposed to TNF- α (50 ng/ml) for different time

intervals (0, 5, 15, 30, 45, and 60 minutes). Lipids were extracted, and DAG and ceramide contents were determined as described (*i.e.* optical density) in Example 7. Results were measured as the mean \pm S.D. of three different studies. It was found that in astrocytes, the DAG content was much higher than the ceramide content.

5 Stimulation of cells with TNF- α resulted in a time-dependent increase in the production of ceramide (about 3 fold after 45 min). In contrast to induction of ceramide production, the level of DAG, an activator of protein kinase C and acidic sphingomyelinase, was unchanged at different time points of stimulation. Similar to TNF- α (FIG. 6), stimulation of astrocytes with IL-1 β for different times also induced a significant increase in the ceramide content (FIG. 7).
10 Almost three to four fold increase in ceramide production was found in astrocytes after 30 or 45 min of exposure with TNF- α or IL-1 β . This increase in ceramide was paralleled by TNF- α - and IL-1 β -induced decrease in sphingomyelin (FIG. 6 and FIG. 7). Sphingomyelin concentration decreases of approximately 18 to 25% could be observed as early as 15 min following treatment of astrocytes (FIG. 6 and FIG. 7) and maximal effects of up to 45 to 50%
15 SM hydrolysis were observed after 30 to 45 min of treatment with TNF- α or IL-1 β . These results indicate that both TNF- α and IL-1 β modulate the degradation of sphingomyelin to produce ceramide, the putative second messenger of the sphingomyelin signal transduction pathway, in rat primary astrocytes within a short time. Interestingly, it was found that treatment of astrocytes with antioxidants like NAC or PDTC 1 h before the addition of TNF- α or IL-1 β
20 potentially blocked the decrease in sphingomyelin as well as the increase in ceramide (FIG. 6 and FIG. 7) indicating that reactive oxygen species (ROS) are possibly involved in cytokine-induced degradation of SM to ceramide.

TNF- α and IL-1 \sim decrease intracellular level of reduced glutathione (GSH) in rat primary astrocytes and NAC blocks this decrease Since intracellular level of GSH is an
25 important regulator of the redox state of a cell, to understand the relationship between induction of ceramide production and intracellular level of GSH in cytokine-stimulated astrocytes, rat primary astrocytes were stimulated with TNF- α or IL-1 β and the level of GSH was measured at different times. Cells preincubated with 10 mM NAC for 1 h received either TNF- α (50 ng/ml) or IL-1 β (50 ng/ml). At different time intervals (0, 15, 30, 45, 60, 75, and 90 minutes), cells
30 were scrapped off and GSH concentrations (100% value is 210 ± 18.5 nmol/mg protein) were measured as described Example 7. Measurements were done in duplicate. The stimulation of

cells with TNF- α or IL-1 β resulted in an immediate decrease in intracellular level of GSH with the maximal decrease (66 to 70% of control) found within 15 to 30 min of initiation of stimulation and with a further increase in time of incubation, the level of GSH was found to be almost normalized (88 to 95% of control at 90 min). These results show that cytokine stimulation induces rapid, short-term production of oxidants which transiently deplete GSH. However, intracellular level of GSH did not decrease when cells were stimulated with cytokines in presence of NAC indicating that NAC inhibited the cytokine-induced degradation of SM to ceramide by maintaining the normal levels of GSH.

Thiol-depleting agents induce the production of ceramide in rat primary astrocytes

Since NAC, a thiol antioxidant, blocked cytokine-mediated depletion of intracellular level of GSH and breakdown of SM to ceramide, the effect of a thiol-depleting agents [diamide and buthione (S,R)-sulfoximine] on ceramide production was examined. Diamide reduces the intracellular level of GSH by its oxidation to GSSG whereas buthione (S,R)-sulfoximine does so by blocking the synthesis of GSH (Shertzer *et al.*, 1995; Akamatsu *et al.*, 1997). Rat primary astrocytes were preincubated with 10 mM NAC for 1 h received diamide (0.5 mM). At different time intervals (0, 15, 30, 45, and 60) cells were washed with HBSS and scrapped off. Lipids were extracted, and level of ceramide was measured as described in the methods section. Ceramide levels were expressed as -fold change over the level at 0 minutes in this study. Results were measured as the mean \pm S.D. of three different studies. At different time intervals, intracellular level of GSH (100% value was 210 ± 18.5 nmol/mg protein) was measured as described in Example 7. Measurements were done in duplicate. Stimulating rat primary astrocytes with diamide resulted in an immediate decrease in intracellular level of GSH due to rapid consumption of intracellular GSH through its nonenzymatic conversion to the oxidized dimer, GSSG (Shertzer *et al.*, 1995) and marked induction of ceramide production (about 7 fold after 30 min of stimulation) indicating that intracellular level of GSH is the important regulator of degradation of SM to ceramide. Consistent with this conclusion, treatment of cells with NAC blocked diamide-mediated decrease in GSH level and induction of ceramide production. Similar to diamide, buthione (S,R)-sulfoximine also decreased the level of GSH and induced the production of ceramide. Thus the low GSH and/or high intracellular oxidant (ROS) levels induced by cytokines and thiol-depleting agents facilitated the induction of ceramide production, while the normal levels of GSH and/or low ROS induced or maintained

by the addition of NAC blocked the hydrolysis of sphingomyelin to ceramide. Taken together, these results demonstrate that the intracellular levels of GSH and/or ROS regulate the extent to which sphingomyelin is degraded to ceramide and ceramide-mediated signaling cascades are transduced.

5 *Aminotriazole and hydrogen peroxide induce the production of ceramide in rat primary astrocytes* Inhibition of cytokine-mediated induction of ceramide production by antioxidants and induction of ceramide production by thiol-depleting agents alone indicate the involvement of ROS in the induction of ceramide production. Therefore, the effect of exogenous addition of an oxidant, like H_2O_2 , or endogenously produced H_2O_2 on inhibition of catalase with
10 aminotriazole (ATZ), which inhibits endogenous catalase to increase the level of H_2O_2 , on the induction of ceramide production. Rat primary astrocytes were incubated with 5 mM aminotriazole or 0.5 mM H_2O_2 in presence or absence of 10 mM NAC. At different time intervals (0, 15, 30, 45, and 60 minutes), cells were washed with HBSS and scrapped off. Lipids were extracted, and level of ceramide was measured as described in Example 7.
15 Ceramide levels were expressed as -fold change over the level at 0 minutes in these studies. Results are measured as the mean \pm S.D. of three different studies. The time course of ceramide production in rat primary astrocytes following the addition of ATZ. Approximately 45 min following the addition of ATZ, ceramide generation increased more than 5-fold over baseline. However, pretreatment of cells with NAC blocked the ATZ-mediated increase in
20 ceramide production. Consistent with the increase in ceramide production by ATZ, addition of exogenous H_2O_2 to astrocytes also induced the production of ceramide with the maximum increase of about 7-fold after 15 min. These results clearly indicate that intracellular levels of ROS regulate the production of ceramide.

Inhibition of cytokine-mediated production of ceramide in rat primary microglia, oligodendrocytes and C6 glial cells by NAC Since NAC inhibited the cytokine-mediated
25 production of ceramide in rat primary astrocytes, the inventor examined the effect of NAC on cytokine-mediated induction of ceramide production in rat primary oligodendrocytes, microglia and C₆ glial cells was examined. Rat primary microglia, oligodendrocytes and C₆ glial cells were preincubated with 10 mM NAC for 1 h in serum-free DMEM/F-12 received TNF- α (50
30 ng/ml). Cells were washed with HBSS and scrapped off at different intervals (0, 15, 30, 45, and 60 minutes). Lipids were extracted, and ceramide content was measured as described in

Example 7. Ceramide levels were expressed as -fold change over the level at 0 minutes in these studies. Results are mean \pm S.D. of three different studies. The addition of TNF- α to oligodendrocytes, microglia or C₆ glial cells induced the production of ceramide. The increase in ceramide in these cells ranges from 2.5 to 4-fold with highest increase in glial cells and lowest in oligodendrocytes. The ceramide levels peaked in glial cells at 30 min following stimulation and 45 min of stimulation in oligodendrocytes and C₆ glial cells. These results show that similar to astrocytes, the SM cycle is also present in microglia, oligodendrocytes and C₆ glial cells. Consistent with the effect of NAC on the production of ceramides in astrocytes, this antioxidant also potently blocked the TNF- α -induced production of ceramide in microglia, oligodendrocytes and C₆ glial cells indicating that ROS are also involved in cytokine-mediated ceramide production in these cells was examined.

NAC inhibits TNF- α - and diamide-mediated apoptosis in rat primary oligodendrocytes by increasing the intracellular level of GSH and decreasing the production of ceramide: Since cytokine-mediated ceramide production is implicated in apoptosis of different cells including brain cells (Brugg *et al.*, 1996; Wiesner and Dawson, 1996), the effect of NAC on TNF- α - as well as diamide-mediated apoptosis in rat primary oligodendrocytes, as evidenced by electrophoretical detection of hydrolyzed DNA fragments ("laddering") was investigated. To understand the role of intracellular level of GSH in inducing apoptosis, rat primary oligodendrocytes were treated with TNF- α or with diamide, a thiol-depleting agent. Cells preincubated with 10 mM NAC for 1 h received either diamide (0.5 mM) or TNF- α (50 ng/ml). Samples were prepared for control cells, diamide, diamide + NAC, TNF- α , and TNF- α + NAC. After 12 h of incubation, cells were harvested and washed with PBS, and genomic DNA was extracted and run on agarose gels as described in the methods section. Ten micrograms of DNA was loaded in each lane. Levels of ceramide and GSH were measured in homogenates as described in Example 7. Results were determined as the mean \pm S.D. of three different studies. Both TNF- α and diamide decreased the intracellular level of GSH, increased the level of ceramide and induced internucleosomal DNA fragmentation as evident from the typical ladder pattern that was generated. Interestingly, blocking of diamide- as well as TNF- α -mediated decrease in intracellular level of GSH by pre-treatment with NAC inhibited the induction of ceramide formation and DNA fragmentation indicating that intracellular level of GSH regulate apoptosis in oligodendrocytes through ceramide formation.

DNA fragmentation in banked human brains with X-adrenoleukodystrophy (X-ALD) and multiple sclerosis (MS) In the CNS, apoptosis may play an important pathogenetic role in neurodegenerative diseases such as ischemic injury, and white matter diseases (Thompson, 1995; Bredesen, 1995). Both X-adrenoleukodystrophy (X-ALD) and MS are demyelinating diseases with the involvement of proinflammatory cytokines in the manifestation of white matter inflammation. Several studies demonstrating the induction of proinflammatory cytokines at the protein or mRNA level in MS patients' cerebrospinal fluid and brain tissue have established an association of proinflammatory cytokines (TNF- α , IL-1 β , IL-2, IL-6 and IFN- γ) with the inflammatory loci in MS (Maimone *et al.*, 1991; Tepper *et al.*, 1995; Rudick and Ransohoff, 1992). Recent documentation of the presence of TNF- α , IL-1 β and IFN- γ in X-ALD brain have revealed the neuroinflammatory character of this disease (Powers *et al.*, 1992; McGuinness *et al.*, 1997).

Therefore, to understand the underlying relationship among intracellular level GSH, level of ceramide and DNA fragmentation in cytokine-inflamed CNS of X-ALD and MS, the inventor measured the levels of GSH and ceramide in homogenates was measured and the DNA fragmentation in nuclei from brains of patients with X-ALD and MS was also studied. Genomic DNA isolated from nuclei of banked human brains was run on agarose gel and photographed as described Example 7. Ten micrograms of DNA was loaded in each lane. Levels of ceramide (optical density) and GSH (nmol/mg protein) were measured in homogenates as described. Results were determined as the mean \pm S.D. of three different studies. In both X-ALD and MS brain homogenates, the level of GSH was lower (55 to 70% of control) and the level of ceramide was higher (2 to 3 fold) compared to those found in control brains. Consistent with a lower level of GSH and a higher level of ceramide, genomic DNA isolated from nuclei of X-ALD and MS brains when run on agarose gels formed the typical ladder pattern which was absent in both of the normal brains. To confirm apoptosis in brain tissues of X-ALD and MS, paraffin-embedded tissue sections of X-ALD and MS were stained with TdT-mediated fragment end labeling. Terminal deoxynucleotidyl transferase (TdT)-mediated end labeling of 3'-OH ends of DNA fragments on paraffin-embedded tissue sections for control, X-ALD and MS samples was carried out using a commercially available kit from Calbiochem, USA. Consistent with increased DNA fragmentation in isolated nuclei of X-ALD and MS, increased TdT staining on brain sections of X-ALD and MS was observed as

compared to those of controls. These observations indicate that intracellular level of GSH are an important factor in cytokine-mediated degradation of SM to ceramide and apoptosis in inflammatory demyelinating diseases like X-ALD and MS.

5

EXAMPLE 8

Lovastatin and Phenylacetate Inhibit the Induction of Nitric Oxide Synthase and Cytokines in Rat Primary Astrocytes, Microglia, and Macrophages

This study explores the role of mevalonate inhibitors in the activation of NF- κ B and the induction of inducible nitric oxide synthase (iNOS) and cytokines (TNF- α , IL-1 β , and IL-6) in
10 rat primary astrocytes, microglia, and macrophages.

Materials and Methods

Reagents Recombinant rat IFN- γ , DMEM/F-12 medium, FBS, and HBSS were from GIBCO-BRL (Gaithersburg, MD). Human IL-1 β was from Genzyme Corp. (Boston, MA). Mouse recombinant TNF- α was obtained from Boehringer Mannheim (Mannheim, Germany).
15 Lovastatin, mevastatin, and farnesyl pyrophosphate were from BIO-MOL Res. Labs Inc. (Plymouth Meeting, PA). Mevalonate, cholesterol, ubiquinone, arginase and LPS (*Escherichia coli*, serotype 0111:B4) were from Sigma Chemical Co. (St. Louis, MO). *N*^G-methyl-L-arginine (L-NMA), FPT inhibitor II, and antibodies against mouse macrophage iNOS were obtained from Calbiochem Corp. (La Jolla, CA). Immunoassay kits for TNF- α ,
20 IL-1 β , and IL-6 were obtained from R&D Systems, Inc. (Minneapolis, MN). NF- κ B DNA binding protein detection kit was from GIBCO-BRL. [γ -³²P]ATP (3,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). [2-¹⁴C]acetate was purchased from ICN Biomedicals Inc. (Irvine, CA). NaPA was prepared from phenylacetic acid (Sigma Chemical Corp.) and NaOH as described (Samid *et al.*, 1992).

25 *Induction of NO production in rat astrocytes, microglia, and C₆ glial cells* Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (McCarthy and DeVellis, 1980). Cells were maintained in DMEM/F-12 medium containing 10% FBS. After 10 d of culture, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure complete removal of the oligodendrocytes and
30 microglia, the shaking was repeated twice after a gap of 1 or 2 d. Cells were trypsinized,

subcultured, and stimulated with LPS or different cytokines in serum-free DMEM/F-12 medium. Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (Giulian and Baker, 1986). In brief, on days 7-9 the mixed glial cultures were washed three times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37°C on a rotary shaker. The floating cells were washed, seeded onto plastic tissue culture flasks, and incubated at 37°C for 2 h. The attached cells were removed by trypsinization and seeded onto new plates for further studies. 90-95% of this preparation was found to be positive for nonspecific esterase, a marker for macrophages and microglia. For induction of NO production, cells were stimulated with LPS or cytokines in serum-free condition. C₆ glial cells obtained from American Type Culture Collection (Rockville, MD) were also maintained and induced with different stimuli as indicated above.

Isolation of rat macrophages and induction of NO production Resident macrophages were obtained from rat by peritoneal lavage with sterile RPMI 1640 medium containing 1% FBS and 100 µg/ml gentamicin. Cells were washed three times with RPMI 1640 at 4°C, and were maintained at 37°C in a humified incubator containing 5% CO₂ in air. Macrophages at a concentration of 2×10^6 /ml in RPMI 1640 medium containing L-glutamine and gentamicin were added in volumes of 800 µl to a 35-mm plate. After 1 h, nonadherent cells were removed by washing, and 800 µl of serum-free RPMI 1640 medium with various stimuli was added to the adherent cells. After 24 h the culture supernatants were transferred to measure NO production.

Cell viability Cytotoxic effects of the inhibitors were determined by measuring the cell viability by Trypan blue exclusion.

Assay for NO synthesis Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. In brief, 400 µl of culture supernatant was allowed to react with 200 µl of Griess reagent (Feinstein *et al.*, 1994), and was incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay. Protein was measured (Bradford, 1976).

Incorporation of [¹⁴C]acetate into cholesterol Astrocytes grown in 100-mm plates (~80% confluency) and preincubated in serum-free media with lovastatin or NaPA for 8 h

received [2-¹⁴C]acetate (10 μ Ci/plate). After 3 h, the cells were washed twice with PBS and scraped off. The lipids were extracted with 1 ml 75% ethanol, and the ethanol extract was saponified with 1 ml of 20% ethanolic KOH at room temperature. To this, 2 ml of water was added, mixed, and extracted twice with 2 ml of hexane. The hexane extracts were dried under
5 nitrogen, dissolved in 50 μ l of CHCl₃/MeOH (1:1), spotted on a TLC plate along with standard [³H]cholesterol, and run with hexane/ ether/acetic acid (70:30:1). The plate was then exposed to a photographic film that was stored at -20°C and developed after 2 d. The lanes corresponding to standard cholesterol were scraped and counted in 5 ml of scintillation fluid.

Immunoblot analysis for iNOS After a 24 h incubation in the presence or absence of
10 different stimuli, cells were scraped off, washed with Hank's buffer, and homogenized in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin A, and 5 μ g/ml leupeptin). After electrophoresis the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and [¹²⁵I]-labeled protein A.

RNA isolation and Northern blot analysis Cells were taken out from culture dishes
15 directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc., Houston, TX), and total RNA was isolated according to the manufacturer's protocol. For Northern blot analyses, 20 μ g of total RNA was electrophoresed on 1.2% denaturing formal-dehyde-agarose gels, electrotransferred to Hybond-Nylon Membrane (Amersham Corp.), and hybridized at 68°C
20 with ³²P-labeled cDNA probe using Express Hyb hybridization solution (Clontech, Palo Alto, CA) as described by the manufacturer. The cDNA fragment for iNOS was amplified by PCRTM using two primers (forward primer: 5'-CTCCTTCAAAGAGGCAAAAATA-3' (SEQ ID NO:1); reverse primer: 5'-CACTTCCTCCAGGATGTTGT-3' (SEQ ID NO:2)), and was cloned in pGEM-T vector (Geller *et al.*, 1993). The clone was confirmed by DNA sequencing,
25 and the insert was used as probe. After hybridization, filters were washed two to three times in solution I (2 \times SSC, 0.05% SDS) for 1 h at room temperature, followed by solution II (0.1 \times SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed with x-ray films (Eastman Kodak Co., Rochester, NY). Same filters were stripped and rehybridized with probes for GAPDH. The relative mRNA content for iNOS was measured after scanning
30 the bands with a BioRad (Model GS-670; Richmond, CA) imaging densitometer.

Nuclear run-on assay For the measurement of gene transcription, nuclei were prepared, and *in vitro* transcriptional activity was measured with nuclei (25×10^6 nuclei per assay) using 30 μCi of [α - ^{32}P]-UTP (400 Ci/mmol) as described (Caira *et al.*, 1995). In brief, the filters were prehybridized in 1 ml of hybridization buffer (50% formamide, $5 \times \text{SSC}$, 1% SDS, 15% dextran sulfate, $1 \times \text{Denhardt's}$ solution, and 50 $\mu\text{g/ml}$ heparin). After 24 h of prehybridization in the above buffer, hybridization was carried out with the labeled RNAs (1.3×10^5 cpm) at 42°C for 60 h to 3 μg of the immobilized plasmid pGEM-T as a control, or to plasmids containing inserts of rat glyceraldehyde-3-phosphate dehydrogenase, rat actin, and human iNOS cDNAs. The filters were washed twice in $2 \times \text{SSC}$, 0.1% SDS for 15 min at SAC, and twice in $0.5 \times \text{SSC}$, 0.1% SDS for 15 min. Then the filters were treated with RNase buffer (300 mM NaCl, 10 mM TrisHCl, pH 7.4, 40 mM EDTA, 10 $\mu\text{g/ml}$ RNase A, and 350 U/ml RNase T1) at 37°C for 30 min, in the same buffer without RNases for another 30 min, and were then autoradiographed.

Determination of TNF- α , IL-1 β , and IL-6 in culture supernatants Cells were stimulated with LPS in serum-free media for 24 h in the presence or absence of lovastatin or NaPA, and concentrations of TNF- α , IL-1 β , and IL-6 were measured in culture supernatants by using high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Inc.) according to the manufacturer's instructions.

Preparation of nuclear extracts and electrophoretic mobility shift assay Nuclear extracts from stimulated or unstimulated astrocytes (1×10^7 cells) were prepared (Dignam *et al.*, 1983) with slight modifications. Cells were harvested, washed twice with ice-cold PBS, and lysed in 400 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 2 mM MgCl_2 , 0.5 mM DTT, 1 mM PMSF, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ pepstatin A, and 5 $\mu\text{g/ml}$ leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μl of buffer B (20 mM Hepes, pH 7.9, 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ pepstatin A, and 5 $\mu\text{g/ml}$ leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μl of modified buffer C (20 mM Hepes, pH 7.9, 20% [vol/vol] glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and stored at -70°C until use. Nuclear

extracts were used for the electrophoretic mobility shift assay using the NF- κ B DNA binding protein detection system kit (GIBCO/BRL) according to the manufacturer's protocol.

Results

Inhibitors of mevalonate pathway inhibit LPS-induced expression of iNOS in primary astrocytes Both HMG-CoA reductase and mevalonate pyrophosphate decarboxylase are the rate-limiting enzymes of the mevalonate pathway (Goldstein and Brown, 1990; Castillo *et al.*, 1991). The inventor examined the effect of inhibitors of HMG-CoA reductase (lovastatin and mevastatin) and mevalonate pyrophosphate decarboxylase (NaPA) on the induction of iNOS and production of NO. Results in Table 15 show that bacterial LPS at a concentration of 1.0 μ g/ml induced the production of NO by about eightfold. Inhibition of NO production by arginase, an enzyme that degrades the substrate (L-arginine) of NOS and L-NMA, a competitive inhibitor of NOS, indicates that LPS-induced NO production in astrocytes is dependent on NOS-mediated arginine metabolism (Table 15). Lovastatin or mevastatin alone was neither stimulatory nor inhibitory to nitrite production in control astrocytes. Both the inhibitors, however, when added 8 h before the addition of LPS, potentially inhibited LPS-mediated induction of nitrite production in astrocytes. Only 25% inhibition in LPS-induced NO production was found when lovastatin was added to the cells along with LPS, however, the degree of inhibition increased with the increase in time of preincubation, with lovastatin reaching about 90% inhibition of NO production within 8-10 h of preincubation.

To understand the mechanism of inhibitory effect of these inhibitors on LPS-mediated nitrite production, we examined the effect on protein and mRNA levels of iNOS. Rat primary astrocytes were preincubated in serum-free media with different concentrations of lovastatin (5 or 10 μ M) or NaPA (2 or 5 mM) or a combination of 2 μ M lovastatin and 2 mM NaPA for 8 h received 1.0 μ g/ml of LPS. After 24 h, supernatants were used for nitrite assay as described in the Methods section of this example. Data was measured as the mean \pm SD of three different experiments. Cell homogenates were electrophoresed, transferred onto nitrocellulose membranes, and immunoblotted with antibodies against mouse macrophage iNOS as described in Methods. Samples tested included control, LPS, LPS + lovastatin (5 μ M), LPS + lovastatin (10 μ M), LPS + NaPA (2 mM), LPS + NaPA (5 mM), and LPS + lovastatin (2 μ M) + NaPA (2 mM). After 5 h of incubation, cells were taken out directly by adding ultraspec-II RNA reagent (Biotecx Laboratories Inc.) to the plates for isolation of total RNA, and Northern blot analysis

for iNOS mRNA was carried out as described in Methods. Western blot analysis with antibodies against murine macrophage iNOS and Northern blot analysis for iNOS mRNA analysis of LPS-stimulated astrocytes clearly showed that both lovastatin and NaPA significantly inhibited the LPS-mediated induction of iNOS protein and mRNA. A combination of lovastatin and NaPA at a dose lower than the one used individually almost completely inhibited LPS-induced production of NO and expression of iNOS.

To gain further insight into the mechanism of the inhibitory effect of lovastatin and NaPA on LPS-mediated expression of iNOS mRNA, the inventor examined the influence of lovastatin and NaPA on the rate of iNOS gene transcription, as measured by nuclear run-on assays. Rat primary astrocytes preincubated in serum-free media with 10 μ M lovastatin or 5 mM NaPA, or a combination of 2 μ M lovastatin and 2 mM NaPA for 8 h received 1.0 μ g/ml of LPS. After 4 h cells were taken out, and nuclei were collected for nuclear run-on assays. 32 P-labeled mRNA was transcribed *in vitro* from isolated nuclei, and 1.3×10^5 cpm of run-on products were hybridized to each blot as described in Methods. The plasmids used were pGEM-T without any insert (negative control) or containing iNOS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or actin cDNA inserts. Samples tested included control, LPS, LPS +lovastatin (10 μ M), LPS + NaPA (5 mM), and LPS + lovastatin (2 μ M) + NaPA (2 mM). LPS induced the transcription of the iNOS gene in astrocytes, and that preincubation of cells with lovastatin or NaPA inhibited the relative rate of LPS-induced nuclear transcription of the iNOS gene. Consistent with the inhibition of LPS-induced expression of mRNA, protein and activity of iNOS by lovastatin and NaPA, the combination of lovastatin and NaPA completely inhibited the transcription of iNOS gene. These results clearly indicate that lovastatin and NaPA inhibit LPS-induced expression of iNOS mRNA, protein and activity by inhibiting transcription of the iNOS gene.

Table 15
Inhibition of LPS-induced NO Production in Rat Primary Astrocytes
by Lovastatin and Mevastatin

Stimuli	Nitrite nmol/mg/24 h	% Inhibition
Control	2.9±0.5	--
LPS	25.3±3.2	--
LPS + arginase	5.9±0.8	87
LPS + L-NMA	5.5±0.7	88
Lovastatin	2.9±0.3	--
Mevastatin	2.8±0.4	--
LPS + lovastatin	5.2±0.5	90
LPS + mevastatin	5.5±0.5	88

Astrocytes were cultured for 24 h in serum-free DMEM/F-12 with the listed reagents; nitrite concentration in the supernatants was measured as described in Methods. Arginase (100 U/ml) and L-NMA (0.1 mM) were added to the cells together with LPS (1.0 µg/ml), however, cells preincubated with lovastatin (10 µM) or mevastatin (10 µM) for 8 h received LPS. Data are mean±SD of three different experiments.

To determine if the synergistic inhibitory effect of lovastatin and NaPA on LPS-induced iNOS expression in astrocytes could be explained solely by inhibition of the mevalonate pathway, the inventor examined the incorporation of [2-¹⁴C]acetate into cholesterol. Cells preincubated with lovastatin or NaPA for 8 h received [2-¹⁴C]acetate for 3 h. Lovastatin (10 µM) and NaPA (5 mM) inhibited the synthesis of cholesterol by 73±6.2 and 64±5.3%, respectively. The combination of lovastatin (2 µM) and NaPA (2 mM), however, caused 93±4.2% inhibition, indicating that lovastatin and NaPA affect cholesterol synthesis in an additive fashion. Therefore, absence of complete inhibition of iNOS mRNA or protein by lovastatin or NaPA could be due to the absence of complete inhibition of the mevalonate pathway and depletion of mevalonate metabolites.

Inhibition of LPS- and cytokine-induced production of NO by lovastatin in rat primary astrocytes Similar to LPS, different cytokines and their several combinations are known to

induce the expression of iNOS (Jaffrey and Synder, 1995; Mitrovic *et al.*, 1994; Bo *et al.*, 1994; Merrill *et al.*, 1993). To examine whether cytokine-induced NO production is also inhibited by lovastatin, primary astrocytes were stimulated with different combinations of LPS, TNF- α , IL-1 β , and IFN- γ for 24 h, and the production of NO was measured. Rat primary astrocytes preincubated in serum-free media with 10 μ M lovastatin for 8 h received different combinations of LPS and cytokines. After 24 h of incubation, production of nitrite was measured in supernatants as described earlier. Data was measured as the mean \pm SD of three different experiments. Cell homogenates were analyzed for iNOS protein by immunoblotting technique as described before. Concentration of different stimuli were as follows: LPS, 0.5 μ g/ml; TNF- α , 20 ng/ml; IL-1 β , 50 ng/ml; IFN- γ , 50 U/ml. Samples that were assayed included control, LPS + TNF- α , LPS + IFN- γ , TNF- α + IL-1 β , TNF- α + IFN- γ , LPS + TNF- α + lovastatin, LPS + IFN- γ + lovastatin, TNF- α + IL-1 β + lovastatin, and TNF- α + IFN- γ + lovastatin. All the combinations of LPS and cytokines significantly induced production of NO, however, addition of 10 μ M lovastatin to astrocytes potently inhibited NO production and induction of iNOS protein, indicating that similar to LPS, cytokine-mediated expression of iNOS also involves the mevalonate pathway. Under similar conditions, lovastatin was also found to inhibit LPS-and cytokine-induced NO production in rat C₆ glial cells.

Inhibition of LPS-induced activation of NF-k β and expression of iNOS by lovastatin and NaPA, and its reversal by farnesyl pyrophosphate in rat primary astrocytes. Since activation of NF-k β is necessary for induction of iNOS (Xie *et al.*, 1994; Kwon *et al.*, 1995), to understand the basis of the inhibition of iNOS, the inventor examined the effect of these inhibitors on LPS-induced activation of NF-k β in astrocytes by gel-shift DNA-binding assay. Rat primary astrocytes incubated in serum-free media received 1.0 μ g/ml of LPS. After 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF-k β as described in Methods. Samples tested included control, LPS, LPS-treated nuclear extract with 25-fold excess of unlabeled probe, and LPS-treated nuclear extract with a 50-fold excess of unlabeled probe, respectively. Treatment of astrocytes with 1.0 μ g/ml of LPS resulted in activation of NF-k β . This gel-shift assay detected a specific band in response to LPS that was competed off by an unlabelled probe. Lovastatin or NaPA alone at different concentrations failed to induce NF-k β .

In an additional experiment, cells preincubated in serum-free media with 10 μ M of lovastatin or 5 mM of NaPA for 8 h received 1.0 μ g/ml of LPS. Samples tested included control, LPS, LPS + lovastatin (5 μ M), LPS + lovastatin (10 μ M), LPS + NaPA (2 mM), LPS + NaPA (5 mM). Both lovastatin and NaPA, however, markedly inhibited LPS-induced
5 activation of NF- κ B, indicating that inhibition of iNOS expression by lovastatin and NaPA is possibly due to inhibition of NF- κ B.

The inventor has demonstrated earlier that activation of NF- κ B is necessary for iNOS expression in rat primary astrocytes, and that cAMP derivatives inhibit the expression of iNOS by inhibiting the activation of NF- κ B. To evaluate the possible mechanism of the effect of
10 lovastatin and NaPA, or to determine whether reduced concentrations of end products as opposed to intermediate products of the mevalonate pathway were responsible for the effects of lovastatin and NaPA, the inventor performed rescue experiments with cholesterol, ubiquinone, mevalonate, and FPP. Cells preincubated in serum-free media with 10 μ M of lovastatin or 5 mM of NaPA for 8 h received 1.0 μ g/ml of LPS along with 100 μ M mevalonate or 200 μ M
15 farnesyl pyrophosphate. After 24 h, supernatants were used for nitrite assay as described in Methods. Data was measured as the mean \pm SD of three different experiments. Samples tested included control, LPS, LPS + lovastatin, LPS + lovastatin + mevalonate, LPS + lovastatin + FPP, LPS + NaPA, LPS + NaPA + mevalonate, LPS + NaPA + FPP. After 5 h of incubation, cells were analyzed for iNOS mRNA by Northern blotting technique as described earlier. After
20 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF- κ B as described in Methods. The addition of 10 μ M ubiquinone or cholesterol to astrocytes did not prevent the inhibitory effect of lovastatin and NaPA. NF- κ B and iNOS were induced in the LPS, LPS + lovastatin + mevalonate, LPS + lovastatin + FPP, and LPS + NaPA + FPP treated cells. These observations
25 support the possibility that depletion of intermediary products rather than end products of mevalonate pathway is responsible for the observed inhibitory effect of lovastatin or NaPA on LPS-induced iNOS expression. On the other hand, mevalonate or FPP substantially reversed the inhibitory effect of lovastatin on iNOS expression and NF- κ B activation. FPP, however, but not mevalonate, reversed the inhibitory effect of NaPA, indicating that the use of mevalonate
30 rather than its synthesis is the prime target of the NaPA.

An inhibitor of Ras farnesyl protein transferase (FPT inhibitor II) inhibits LPS-induced expression of iNOS and activation of NF- κ B in rat primary astrocytes. FPT inhibitor II selectively inhibits ras farnesyl protein transferase with the IC₅₀ of 75 nM. In whole cells, however, 25-250 μ M of FPT inhibitor II inhibits farnesylation of p21^{ras} by ~ 90% (Manne *et al.*, 1995). Inhibition of LPS-induced expression of iNOS and activation of NF- κ B by NaPA and its reversal by FPP, but not by mevalonate, indicates a possible involvement of the farnesylation reaction in activation of NF- κ B and induction of iNOS. Since farnesylation is a necessary step for activation of p21^{ras}, the central molecule upstream of the Raf/MAP kinase cascade, the inventor examined the effect of FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, on LPS-mediated expression of iNOS and activation of NF- κ B in rat primary astrocytes. Rat primary astrocytes preincubated in serum-free media with 100 μ M or 200 μ M FPT inhibitor II for 1 h received 1.0 μ g/ml of LPS. After 24 h of incubation, supernatants were used for nitrite assay as described in Methods. Data were determined as the mean \pm SD of three different experiments. After 5 h of incubation, cells were analyzed for iNOS mRNA by Northern blotting technique as described earlier. After 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF- κ B as described in Methods. Samples tested included control, LPS, LPS + FPT inhibitor II (100 μ M), and LPS + FPT inhibitor II (100 μ M). Preincubation of cells for 1 h with 100 or 200 μ M FPT inhibitor II potentially inhibited LPS-induced activation of NF- κ B, expression of iNOS, and production of NO, demonstrating the importance of p21^{ras} farnesylation in LPS-mediated activation of NF- κ B and induction of iNOS in astrocytes.

Lovastatin and NaPA inhibit the LPS-induced expression of TNF- α , IL- β , and IL-6 in rat primary astrocytes. Activated astrocytes, the major glial cell population of brain, are reported to secrete TNF- α , IL-1 β , and IL-6 (Sharif *et al.*, 1993). Since lovastatin and NaPA inhibited LPS-induced expression of iNOS in astrocytes, the inventor examined the effect of these two inhibitors on LPS-induced expression of TNF α , IL-1 β , and IL-6. Rat primary astrocytes preincubated in serum-free media with different concentrations of lovastatin (5 or 10 μ M) or NaPA (2 or 5 mM), or a combination of 2 μ M of lovastatin and 2 mM of NaPA for 8 h, received 1.0 μ g/ml of LPS. Samples tested included control, LPS, LPS + lovastatin (5 μ M), LPS + lovastatin (10 μ M), NaPA (2 mM), LPS + NaPA (5 mM), and LPS + lovastatin (2 μ M) + NaPA (2 mM). After 5 h of incubation, cells were analyzed for TNF- α , IL-1 β , and IL-6

mRNAs by Northern blotting technique as described earlier. Astrocytes preincubated with lovastatin or NaPA were stimulated with LPS. Concentrations of TNF α , IL-1 β , and IL-6 were measured in the supernatants after 24 h of incubation (Table 16), and the mRNA expression of these-cytokines was examined in the cells after 5 h of LPS stimulation. Bacterial LPS

5 markedly induced the mRNA expression and production of respective cytokines in astrocytes. Although lovastatin or NaPA alone had no effect on the production of cytokines, however, these two compounds strongly inhibited LPS-induced production of TNF- α , IL-1 β , and IL-6 in the supernatants (Table 16). The decrease in cytokine production was also accompanied by an inhibition of their mRNA expression, demonstrating that lovastatin and NaPA downregulate

10 expression of all the inflammatory mediators (iNOS, TNF- α , IL-1 β , and IL-6) in astrocytes.

Table 16
Inhibition of LPS-induced Production of NO, TNF- α , IL-1 β , and IL-6 in
Rate Primary Astrocytes, Microglia, and Macrophages by Lovastatin and NaPA

Cells	Production of NO or cytokines	Treatments		
		LPS only	LPS + lovastatin	LPS + NaPA
Astrocytes	NO	25.3 \pm 3.2	5.2 \pm 0.4	5.4 \pm 0.6
	TNF- α	5.3 \pm 0.8	0.3 \pm 0.05	0.4 \pm 0.06
	IL-1 β	10.4 \pm 1.5	0.8 \pm 0.1	1.1 \pm 0.2
	IL-6	136.5 \pm 16.8	6.9 \pm 0.9	7.6 \pm 0.8
Microglia	NO	81.2 \pm 6.9	5.9 \pm 0.4	6.9 \pm 0.9
	TNF- α	14.5 \pm 2.1	0.9 \pm 0.1	1.3 \pm 0.2
	IL-1 β	28.2 \pm 3.4	2.1 \pm 0.3	2.4 \pm 0.2
	IL-6	295.6 \pm 33.5	7.8 \pm 1.1	9.3 \pm 1.2
Macrophages	NO	118.5 \pm 12.5	7.2 \pm 0.9	9.5 \pm 0.7
	TNF- α	18.6 \pm 2.3	1.2 \pm 0.1	1.7 \pm 0.2

Table 16 - Continued

Cells	Production of NO or cytokines	Treatments		
		LPS only	LPS + lovastatin	LPS + NaPA
	IL-1 β	34.6 \pm 4.5	2.3 \pm 0.3	3.1 \pm .4
	IL-6	350.0 \pm 27.6	8.3 \pm 0.6	10.2 \pm 1.4

Cells preincubated with 10 μ M lovastatin or 5 mM NaPA for 8 h in serum-free condition was stimulated with 1.0 μ g/ml of LPS. After 24 h of incubation, concentrations of NO, TNF- α , IL-1 β , and IL-6 were measured in supernatants as described in Methods. NO is expressed as nmol/24 h/mg protein whereas TNF- α , IL-1 β , and IL-6 are expressed as ng/24 h/mg protein. Data are expressed as the mean \pm SD of three different experiments.

Inhibition of LPS-induced production of NO, TNF- α , IL-1 β , and IL-6 in rat primary microglia and macrophages by lovastatin. Both macrophages and microglia, important sources of NO and cytokines, actively participate in the pathophysiologies of different inflammatory disorders. Since lovastatin and NaPA inhibited the LPS-induced production of NO, TNF- α , IL-1 β , and IL-6 in astrocytes, the inventor also examined the effect of these two compounds on LPS-stimulated production of NO, TNF- α , IL-1 β , and IL-6 in rat primary microglia and macrophages (Table 16). The rate of production of NO and cytokines after LPS stimulation was much higher in both macrophages and microglia than in astrocytes. Similar to astrocytes, lovastatin or NaPA alone had no effect on the production of NO and cytokines in macrophages and microglia. Both of these compounds, however, strongly inhibited the LPS-induced production of NO, TNF- α , IL-1 β , and IL-6 in macrophages and microglia (Table 16). These studies demonstrate the importance of the mevalonate pathway in the LPS induced production of NO, TNF- α , IL-1 β , and IL-6 in astrocytes as well as in microglia and macrophages (Table 16). The inhibitors (lovastatin, mevastatin, or NaPA), cytokines (TNF- α , IL-1 β , and IFN- γ), or LPS used under these experimental conditions had no effect on the viability of astrocytes, microglia, or macrophages, measured by Trypan blue exclusion. Therefore, the conclusion drawn in this study is not due to any change in viability of the cells.

Discussion

Several lines of evidence presented herein clearly support the conclusion that inhibitors of HMG-CoA reductase (lovastatin or mevastatin) and NaPA reduce the induction of inflammatory mediators (iNOS, TNF- α , IL-1 β , and IL-6) in rat astrocytes, microglia, and macrophages, demonstrating the involvement of mevalonate metabolite(s) and farnesyl pyrophosphate in the induction of inflammatory mediators. This conclusion was based on the following observations: first, LPS-induced expression of iNOS, TNF- α , IL-1 β , and IL-6, and activation of NF-k β , was inhibited by lovastatin and NaPA; second, inhibitory effects of lovastatin and NaPA on LPS-mediated induction of iNOS and cytokines was not reversed by cholesterol and ubiquinone, end products of mevalonate pathway, indicating that this inhibitory effect of lovastatin was not due to depletion of end products of mevalonate pathway; third, the reversal of inhibitory effect of lovastatin by mevalonate and FPP and that of NaPA by only FPP, but not by mevalonate, indicates that mevalonate and FPP are necessary compounds for LPS signal transduction; fourth, inhibition of LPS-induced activation of NF-k β and induction of iNOS by FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, indicates that farnesylation of p21^{ras} or other proteins is required for signal transduction in the LPS-induced expression of iNOS. Since iNOS, TNF- α , IL-1 β , and IL-6 have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases (Mitrovic *et al.*, 1994; Merrill *et al.*, 1993), the inventor's results provide a potentially important mechanism whereby inhibitors of HMG-CoA reductase and mevalonate pyrophosphate decarboxylase may ameliorate neural injury. Inhibition of LPS-induced NF-k β activation and iNOS expression by lovastatin, NaPA, and FPT inhibitor II indicates that the observed inhibition of iNOS expression is due to inhibition of NF-k β activation.

Since mevalonate availability regulates the posttranslational isoprenylation of many intracellular signaling proteins including p21^{ras} (Goldstein and Brown, 1990), the observed inhibition of NF-k β activation and induction of iNOS by lovastatin and NaPA may be due to the decrease or lack of the isoprenylation of p21^{ras}, that in turn leads to the lack of or abnormal signal transmission from receptor tyrosine kinase to Raf/MAP kinase cascade, activation of NF-k β , and induction of iNOS. The prerequisite of Ras farnesylation in transduction of signals from receptor tyrosine kinase to Raf/MAP kinase cascade indicates a possible role of metabolites of mevalonate pathway in the modulation of iNOS induction.

NO, a diffusible free radical, plays many roles as a signaling and as an effector molecule in diverse biological systems including neuronal messenger, vasodilation, and antimicrobial and antitumor activities (Nathan, 1992; Jaffrey and Synder, 1995). In the nervous system, NO appears to have both neurotoxic and neuroprotective effects, and may have a role in the pathogenesis of stroke and other neurodegenerative diseases, and in demyelinating conditions (e.g., multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) associated with infiltrating macrophages and production of proinflammatory cytokines (Mitrovic *et al.*, 1994; Merrill *et al.*, 1993; Dawson *et al.*, 1991). NO and peroxynitrite (reaction product of NO and O₂⁻) are potentially toxic molecules to neurons and oligodendrocytes that may mediate toxicity through the formation of iron-NO complexes of iron-containing enzyme systems (Drapier and Hibbs, 1988), oxidation of protein sulfhydryl groups (Radi *et al.*, 1991), nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (Wink *et al.*, 1991). Although monocytes/macrophages are the primary source of iNOS in inflammation, LPS and other cytokines induce a similar response in astrocytes and microglia (Hu *et al.*, 1995; Galea *et al.*, 1992). NO derived from macrophages, microglia, and astrocytes has been implicated in the damage of myelin-producing oligodendrocytes in demyelinating disorders like multiple sclerosis and neuronal death during neuronal degenerating conditions including brain trauma (Hu *et al.*, 1995; Merrill *et al.*, 1993). The studies described herein indicate that lovastatin and NaPA, alone or in combination, may represent a possible avenue of research for therapeutics directed against cytokine- and nitric oxide-mediated brain disorders, particularly in demyelinating conditions.

EXAMPLE 9

Amelioration of Experimental Allergic Encephalomyelitis by inhibiting the induction of NOS-2 and proinflammatory cytokines

Proinflammatory cytokines and inducible nitric oxide synthase (iNOS) are involved in the pathogenesis of experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). In the present study the inventor reports the use of N-acetylcysteine (NAC), NaPA and lovastatin as therapeutic agents for the amelioration of the autoimmune demyelinary disease in EAE. The development of demyelinating lesions in EAE or MS is the result of a complex chain of events that involves recognition of specific antigen, T cell

activation, recruitment of nonspecific cells to the lesion, release of numerous cytokines and inflammatory mediators (*e.g.*, NO) by resident glial cells and infiltrating cells, which in turn leads to demyelination and CNS damage. NAC, a potent antioxidant, blocks the induction of iNOS and TNF- α in rat peritoneal macrophages, astrocytes and C₆ glioma. Lovastatin, an inhibitor of the rate limiting enzyme of the mevalonate pathway, has also been shown to block the induction of iNOS and proinflammatory cytokines (TNF- α , IL-1 β and IL-6) in rat astrocytes, microglia and macrophages. The inventor provides evidence that NAC, NaPA or lovastatin inhibits the induction of proinflammatory mediators (TNF- α , IFN- γ and iNOS) in EAE central nervous system and also ameliorate the clinical symptoms of the EAE disease.

10 Materials and Methods

Reagents Female Lewis rats were purchased from Charles River Breeding Laboratories, Wilmington, MA, USA. Myelin basic protein (MBP), complete Freund's adjuvant (CFA), N-acetylcysteine (NAC) and FITC conjugated anti-mouse IgG were purchased from Sigma Chemical Co., USA. Lovastatin was obtained from Calbiochem, USA.

- 15 *Induction and clinical assessment of Experimental allergic encephalomyelitis (EAE)*
Female Lewis rats, 250-300 g, were housed in rat cages and provided with food and water *ad libitum*. Rats were induced with EAE by injecting intradermally 50 μ g of myelin basic protein (MBP) per animal emulsified in complete Freund's adjuvant (CFA) into the medial footpad of each hind leg on day 1 followed by a booster injection on the 7th day under ether anesthesia.
- 20 Clinical symptoms in these rats manifest as an ascending paralysis resulting in death in most animals. The signs of EAE were scored as: (0) normal; (1) piloerection; (2) loss in tail tonicity; (3) hind leg paralysis; (4) paraplegia; and (5) moribund.

- Drug Treatment Regiment* Lovastatin, NAC or NaPA therapy was started on the first day of immunization (day 1) and continued daily for the duration of the study. Lovastatin, NAC or NaPA was dissolved in saline and pH was adjusted to 7.0. One group of rats induced for EAE were given i.p. injection of Lovastatin (2 mg/Kg body weight) and another group of rats induced for EAE were given i.p. injection of NAC (150 mg/Kg body weight) or NaPA. One group of animals induced for EAE was left untreated while another group of animals was not induced for EAE and used as the control group.

- 30 *Immunohistochemistry* Brains were fixed in 10% buffered formalin (Stephens Scientific, Riverdale, NJ). The tissues were embedded in paraffin and sectioned at 4 μ m.

Sections were then stained for various cytokines and cell markers as described below. For single-label immunohistochemistry, sections were incubated with either anti-iNOS antibody (1:100, rabbit polyclonal, Calbiochem, LaJolla, CA) or anti-TNF- α antibody (1:100, rabbit polyclonal, Genzyme, Cambridge, MA) or anti-IFN- γ antibody (1:200, rabbit polyclonal, Biosource International, Camarillo, CA) essentially as described for other antibodies (Hooper *et al.*, 1997). The tissue sections were further incubated with FITC conjugated anti-rabbit IgG (1:100, Sigma, St. Louis, MO), mounted with mounting media (EMS) and analyzed by immunofluorescence microscopy (Olympus) using Adobe photoshop software. For immunofluorescent double-labeling, sections were incubated first with anti-iNOS (1:100) followed by macrophage marker ED1 (1:100, mouse monoclonal, Biosource International, Camarillo, CA). Anti-iNOS was visualized using TRITC conjugated anti-rabbit IgG - (1:100, Sigma, St. Louis, MO) and ED1 using FITC conjugated anti-mouse IgG (1:100, Sigma, St. Louis, MO). Negative control sections were incubated with FITC or TRITC conjugated IgG without the primary antibody.

15 Results

Expression of iNOS, TNF- α and IFN- γ in Lewis rat brain sections of control, EAE and drug-treated animals iNOS in the CNS of Lewis rats was detected by immunofluorescence. Brain sections of control, EAE, EAE treated with NAC, EAE treated with NaPA or EAE treated with lovastatin were immunostained for iNOS as described under materials and methods. Brain sections of rats with EAE show expression of iNOS protein as green fluorescence in a significant number of cells as compared to control. Moreover, treatment of rats with NAC, lovastatin or NaPA, blocked the ability of MBP to induce the expression of iNOS. NAC treatment seems to be better than lovastatin or NaPA in blocking the induction of iNOS.

TNF- α in the CNS of Lewis rats was detected by immunofluorescence. Brain sections of control, EAE, EAE treated with NAC, EAE treated with NaPA and EAE treated with lovastatin were immunostained for TNF- α as described under materials and methods. Similar to the expression of iNOS, a good number of cells show the expression of TNF- α as green fluorescence in EAE brain as compared to controls. Treatment with NAC, lovastatin or NaPA blocked the induction of TNF- α . In case of TNF- α , better inhibition was observed in brains of rats treated with lovastatin or NaPA.

Lewis rat brain sections were stained immunohistochemically for IFN- γ . Brain sections of control, EAE, EAE treated with NAC, EAE treated with NaPA and EAE treated with lovastatin were immunostained for IFN- γ as described under materials and methods. NAC, lovastatin or NaPA treatments also blocked the induction of IFN- γ in brains of animals
5 challenged with MBP. The demonstration of induction of TNF- α , IFN- γ and iNOS in brains of EAE shows a inflammatory disease process and inhibition of the induction of these cytokines in brains of rats treated with NAC, NaPA or lovastatin indicate that these drugs may be of value in ameliorating the inflammatory disease process in EAE.

Co-localization of iNOS with macrophage/microglial marker ED1 To identify the cell
10 type in the CNS of EAE which express iNOS, the inventor performed immunofluorescence double-labeling study using ED1, a specific marker for macrophage/microglia cells of Lewis rat brain sections. Brain sections of control, EAE, EAE treated with NAC, EAE treated with NaPA and EAE treated with lovastatin were immunostained for iNOS (red) and ED1 (green) as described under materials and methods. Co-expression of iNOS and ED1 that was visualized as
15 yellow/orange was seen only in EAE induced rat brain sections indicating that macrophage/microglia of EAE rat brain express iNOS. Animals induced for EAE and treated with NAC, NaPA or lovastatin showed expression of ED1, however, colocalization of ED1 with iNOS, as seen with EAE sections was not observed. In NAC-, NaPA or lovastatin-treated rat brain sections ED1 expression was observed but not iNOS.

NAC and lovastatin protect against EAE disease in Lewis female rats Since NAC, NaPA and lovastatin inhibited the expression of iNOS and proinflammatory cytokines in activated glial cells (astroglia and microglia) and macrophages and in the CNS of Lewis rats with EAE, the inventor examined the therapeutic potential of NAC, NaPA and/or lovastatin on the disease process of EAE. Administration of Lovastatin in Lewis female rats delays the onset
25 of EAE disease symptoms. Data was taken as average clinical disease scores where 0-normal; 1-piloerection; 2-loss in tail tonicity; 3-hind leg paralysis; 4-paraplegia and 5-moribund. Clinical symptoms of EAE appeared in MBP-treated Lewis female rats (n=9) from 7th day after first immunization. In this model, MBP induced a monophasic acute disease progression resulting in death on 11th day, however, control animals receiving only complete Freund's
30 adjuvant did not show any disease symptoms. On the other hand treatment of MBP-injected rats with NAC (n=9), lovastatin (n=9) or NaPA from first day of immunization protected the

rats from the severity of the disease. Both NAC and lovastatin-treated rats received milder clinical symptoms (highest clinical score was between 2 and 3) and specially lovastatin significantly delayed the onset of first of clinical symptom. These results clearly demonstrate that NAC, NaPA and lovastatin provide protection against neuroinflammatory disease of EAE.

5 Discussion

These studies clearly demonstrate that both NAC, NaPA and lovastatin inhibit the expression of proinflammatory cytokines (TNF- α and IFN- γ) and iNOS in the CNS of Lewis rats with EAE and ameliorate the neuroinflammatory disease process in the central nervous system. Immunohistochemical results show a higher degree of expression of iNOS, tumor
10 necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) in brains of rats with acute monophasic EAE relative to the control animals. Although NAC and lovastatin did not block the clinical symptoms of EAE completely in Lewis rat they significantly reduced the severity of the disease. NAC is a nontoxic drug which has been safely used in humans for more than 30 years. Lovastatin is also approved as an cholesterol-lowering drug for humans. Therefore, inhibition
15 of the expression of proinflammatory cytokines and iNOS in the CNS of EAE rats and amelioration of the EAE disease process by NAC and lovastatin indicates that these drugs may have therapeutic importance in the treatment of neuroinflammatory diseases like MS.

EXAMPLE 10

20 Proinflammatory Cytokine-Mediated Apoptosis In Demyelinating Diseases

In the present study, the inventor examined the possible involvement of ROS in cytokine-mediated activation of sphingomyelin breakdown and ceramide formation in rat primary glial cells.

Materials and Methods

25 *Reagent* DMEM/F-12 and fetal bovine serum (FBS) were from Life Technologies, Inc. Human IL1- β was from Genzyme. Mouse recombinant TNF- α was obtained from Boehringer Mannheim, Germany. Diamide, buthione (SR)-sulfoximine, N-acetylcysteine, and pyrrolidinedithiocarbamate were from Sigma.

Isolation and Maintenance of Rat Primary Microglia, Oligodendrocytes, and Astrocytes
30 Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian

and Baker (1986). Briefly, after 7 days the mixed glial cultures were washed 3 times with DMEM/F-12 containing 10% FBS and subjected to a shake at 240 rpm for 4 h at 37°C on a rotary shaker. The floating cells were washed and seeded onto plastic tissue culture flasks and incubated at 37°C. After 30 min the non-attached cells (mostly oligodendrocytes) were removed by repeated washes, and the attached cells were used as microglia. These cells were seeded onto new plates for further studies. Ninety to ninety-five percent of this preparation was positive for nonspecific esterase, a marker for macrophages and microglia.

After 4 h shaking, the flasks were washed three times to remove the floating cells. Medium with 10% FBS was added, and flasks were subjected to another cycle of shaking for 24 h at 250 rpm. The suspended cells were spun at $200 \times g$ and incubated for 30 min in tissue culture dish. The non-attached or weakly attached cells (mostly oligodendrocytes) were removed and seeded onto polylysine-coated dishes and cultured in medium containing 1% FBS. Ninety-five to ninety-seven percent of these cells were positive for galactocerebroside immunostaining.

Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (1980). After 10 days of culture astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the complete removal of all oligodendrocytes and microglia, the shaking was repeated twice after a gap of 1 or 2 days. Attached cells were trypsinized (1 mM EDTA and 0.05% trypsin in 10 mM Tris-buffered saline, pH 7.4) and distributed into culture dishes. These cells when checked for the astrocyte marker glial fibrillar acidic protein were found to be 95-100% positive. C₆ glial cells obtained from ATCC were also maintained in DMEM/F-12 containing 10% FBS as indicated above.

Brain Tissue Frozen and fixed X-adrenoleukodystrophy and multiple sclerosis brain tissues were obtained from Brain and Tissue Banks for Developmental Disorders, University of Maryland, Baltimore, MD 21201. Two X-ALD brains were from 7- and 9-year-old males, and two MS brains were from 30- and 33-year old females. Control brain for X-ALD studies was from an 8-year-old male, and control brain for MS studies was from a 30-year-old female.

Lipid Extraction Approximately 1.0×10^6 cells were exposed to different cytokines in the presence or absence of antioxidants for different periods, and lipids were extracted according to the methods described by Welsh (1996).

Quantification of Sphingomyelin by High Performance TLC and Densitometry

Sphingomyelin was separated from total lipid extracts by high performance TLC (LHPK plates from Whatman) as described by Ganser *et al.* (1988) for phospholipids with the following modification: the plate was overrun for 30 min during its development and was dried overnight in vacuum desiccator. Sphingomyelin was quantitated by densitometric scanning using Imaging Densitometer (model GS-670; Bio-Rad), and software was provided with the instrument by the manufacturer.

Quantification of Ceramide Levels by Diacylglycerol Kinase Assay

Ceramide content was quantified essentially according to Priess *et al.* (1986) using diacylglycerol (DAG) kinase and [γ - 32 P]ATP. Briefly, dried lipids were solubilized in 20 μ l of an octyl β -D-glucoside/cardioplipin solution (7.5% octyl β -D-glucoside, 5 mM cardioplipin in 1 mM DTPA) by sonication in a sonicator bath. The reaction was then carried out in a final volume of 100 μ l containing the 20- μ l sample solution, 50 mM imidazole HCl, pH 6.6, 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 6.6 μ g of DAG kinase, and 1 mM [γ - 32 P]ATP (specific activity of $1-5 \times 10^5$ cpm/nmol) for 30 min at room temperature. The labeled ceramide-1-phosphate was resolved with a solvent system consisting of methyl acetate:*n*-propyl alcohol:chloroform:methanol, 0.25% KCl in water:acetic acid (100:100:100:40:36:2). A standard sample of ceramide was phosphorylated under identical conditions and developed in parallel. Both standard and samples had identical R_F values (0.46). Quantification of ceramide-1-phosphate was carried out by autoradiography and densitometric scanning using Imaging Densitometer (model GS-670; Bio-Rad). Values are expressed either as arbitrary units (absorbance) or as percent change.

Measurement of GSH (Reduced Glutathione) and GSSG Oxidized Glutathione

Concentration of intracellular reduced GSH was measured using a colorimetric assay kit for GSH from R & D Systems. Briefly, 2×10^6 cells were homogenized in 500 μ l of ice-cold 5% metaphosphoric acid and centrifuged at $3000 \times g$ for 10 min. Supernatants were used to assay GSH using 4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulfate and 30% NaOH at 400 nm. Concentration of GSSG was determined according to the method of Griffith (1980) after derivatization with 2-vinylpyridine for 30 min at room temperature.

Detection of DNA Fragmentation Cells (1×10^6) were pelleted in an Eppendorf tube by centrifugation at 1000 rpm for 5 min, washed with phosphate-buffered saline, pH 7.4,

resuspended gently in 50 μ l of a lysis buffer (200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 40 mM EDTA, pH 8.0, 0.5% SDS, 400 ng of RNase A/ μ l), and incubated at 37°C for 1 h. The lysate received 200 μ l of the digestion buffer (200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% SDS, 125 ng of proteinase K/ μ l). The contents were mixed by inversion several times and then
5 incubated at 50°C for 2 h. An equal volume of a mixture of phenol, pH 8.0, chloroform, and isoamyl alcohol (25:24:1, v/v) was added, gently mixed for 10 min, and stored at room temperature for 2 min. The two phases were separated by centrifugation at 3000 rpm for 10 min. The viscous aqueous phase was transferred to a fresh tube, and the phenol/chloroform extraction was repeated. The aqueous phase was extracted with an equal volume of chloroform,
10 and 1.0 M $MgCl_2$ was added to the aqueous phase to a final concentration of 10 mM. The total DNA was precipitated by the addition of 2 volumes of absolute ethanol with several inversions. DNA was pelleted by centrifugation at 3000 rpm for 15 min, washed with 70% ethanol, and air-dried. The pellet was dissolved in 25 μ l of 10 mM Tris-HCl containing 1.0 mM EDTA, pH 8.0, and electrophoresed in 1.8% agarose gel at 4°C. The gel was stained with ethidium
15 bromide, and DNA-intercalated ethidium fluorescence was photographed on Polaroid film 665 (P/N) using an orange filter. To study DNA fragmentation in banked human brain tissues, brain tissues were gently homogenized in 0.85 M sucrose buffer, and nuclei were purified according to the procedure described previously (Lazo *et al.*, 1991). Total genomic DNA was isolated from the nuclei and electrophoresed as described.

20 *Fragment End Labeling of DNA on Paraffin-embedded Tissue Sections of MS and X-ALD Brains* Fragmented DNA was detected *in situ* by the terminal deoxynucleotidyltransferase-mediated binding of 3'-OH ends of DNA fragments generated in response to apoptotic signals, using a commercially available kit (TdT FragEL™) from Calbiochem. Briefly, paraffin-embedded tissue slides were deparaffinized, rehydrated in
25 graded ethanol, treated with 20 μ g/ml proteinase K for 15 min at room temperature, and washed prior to terminal deoxynucleotidyltransferase staining. After terminal deoxynucleotidyltransferase staining, sections were lightly counterstained with methyl green.

Results

NAC and PDTC Block TNF- α - and IL-1 β -induced Degradation of Sphingomyelin to Ceramide in Primary Rat Astrocytes Rat primary astrocytes were cultured in serum-free media
30 with TNF- α or IL-1 β for different times to quantify the production of ceramide using

diacylglycerol (DAG) kinase. Since DAG kinase phosphorylates both DAG and ceramide using [γ - 32 P]ATP as substrate, both lipids can be quantified in the same assay. Rat primary astrocytes were exposed to TNF- α (50 ng/ml) for different time intervals (0, 5, 15, 30, 45 and 60 minutes). Lipids were extracted, and DAG and ceramide contents were determined as described under "Materials and Methods." Results were determined as the mean \pm S.D. of three different studies. It was found that in astrocytes, the DAG content was much higher than the ceramide content. Stimulation of cells with TNF- α resulted in a time-dependent increase in the production of ceramide (about 3-fold after 45 min). In contrast to induction of ceramide production, the level of DAG, an activator of protein kinase C and acidic sphingomyelinase, was unchanged at different time points of stimulation.

In another experiment, rat primary astrocytes preincubated with either 10 mM NAC or 100 μ M PDTC for 1 h in serum-free DMEM/F-12 received TNF- α (50 ng/ml). At different time intervals (0, 15, 30, 45, and 60 minutes), cells were washed with HBSS and scraped off. Lipids were extracted, and levels of ceramide (100% value is 4.51 ± 0.1 nmol/mg protein) and sphingomyelin (100% value is 25.39 ± 6.27 nmol/mg protein) were measured as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. TNF- α -induced degradation of sphingomyelin to ceramide was inhibited by NAC and PDTC.

Similar to TNF- α , stimulation of astrocytes with IL-1 β for different times also induced a significant increase in the ceramide content. Rat primary astrocytes preincubated with either 10 mM NAC or 100 μ M PDTC for 1 h in serum-free DMEM/F-12 received IL-1 β (50 ng/ml). At different time intervals (0, 15, 30, 45, and 60 minutes), cells were washed with HBSS and scraped off. Lipids were extracted, and levels of ceramide (100% value is 4.51 ± 0.1 nmol/mg protein) and sphingomyelin (100% value is 25.39 ± 6.27 nmol/mg protein) were measured as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. Almost 3-4-fold increase in ceramide production was found in astrocytes after 30 or 45 min of exposure with TNF- α or IL-1 β . This increase in ceramide was paralleled by TNF- α - and IL-1 β -induced decrease in sphingomyelin. Sphingomyelin turnover of approximately 18-25% could be observed as early as 15 min following treatment of astrocytes, and maximal effects of up to 45-50% SM hydrolysis were observed after 30-45 min of treatment with TNF- α or IL 1 β .

These studies indicate that both TNF- α and IL-1 β can modulate the degradation of sphingomyelin to produce ceramide, the putative second messenger of the sphingomyelin signal transduction pathway, in rat primary astrocytes within a short time. Interestingly, the inventor found that treatment of astrocytes with antioxidants like NAC (10 mM) 1 h before the addition of TNF- α or IL-1 β potentially blocked the decrease in sphingomyelin as well as the increase in ceramide, whereas 10 mM acetate had no effect on the degradation of SM to ceramide. Similar to NAC, another antioxidant PDTC also inhibited cytokine-mediated degradation of SM to ceramide. These studies indicate that reactive oxygen species (ROS) are possibly involved in cytokine-induced degradation of SM to ceramide.

TNF- α and IL-1 β Decrease Intracellular Level of Reduced Glutathione (GSH) in Rat Primary Astrocytes and NAC Blocks This Decrease Since the intracellular level of GSH is an important regulator of the redox state of a cell, to understand the relationship between induction of ceramide production and intracellular level of GSH in cytokine-stimulated astrocytes, cells were stimulated with TNF- α or IL-1 β , and the level of GSH was measured at different times (0, 15, 30, 45, 60, 75, and 90 minutes). Rat primary astrocytes preincubated with 10 mM NAC for 1 h received either TNF- α (50 ng/ml) or IL-1 β (50 ng/ml). At different time intervals, cells were scraped off, and GSH concentrations (100% value is 182.5 ± 15.4 nmol/mg protein) were measured as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. The stimulation of cells with TNF- α or IL-1 β resulted in an immediate decrease in intracellular level of GSH with the maximal decrease (66-70% of control) found within 15-30 min of initiation of stimulation, and with a further increase in time of incubation, the level of GSH was found to be almost normal (88-95% of control at 90 min). These studies indicate that cytokine stimulation apparently induces rapid, short term production of oxidants which transiently deplete GSH. However, the lack of decrease of GSH (FIG. 4) and lack of hydrolysis of SM in the presence of NAC in the cytokine-treated cells indicate that NAC inhibited the cytokine-induced degradation of SM to ceramide by maintaining the normal levels of GSH.

Thiol-depleting Agents Induce the Production of Ceramide in Rat Primary Astrocytes Since NAC, a thiol antioxidant, blocked cytokine-mediated depletion of intracellular levels of GSH and breakdown of SM to ceramide, the inventor investigated the effect of thiol-depleting agents (diamide and buthione-(SR)-sulfoximine) on ceramide production. Diamide reduces the

intracellular level of GSH by its oxidation to GSSG, whereas buthione-(SR)-sulfoximine does so by blocking the synthesis of GSH (Shertzer *et al.*, 1995; Akamatsu *et al.*, 1997). Rat primary astrocytes preincubated with 10 mM NAC for 1 h received diamide (0.5 mM). At different time intervals (0, 15, 30, 45, and 60 minutes), cells were washed with HBSS and scraped off. Lipids were extracted, and the level of ceramide (100% value is 4.51 ± 0.1 nmol/mg protein) was measured as described under "Materials and Methods". Results were measured as the mean \pm S.D. of three different studies. Additionally, at different time intervals (0, 15, 30, 45, and 60 minutes), intracellular level of GSH (100% value is 182.5 ± 15.4 nmol/mg protein) was measured as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. Stimulating rat primary astrocytes with diamide resulted in an immediate decrease in intracellular level of GSH due to rapid consumption of intracellular GSH through its nonenzymatic conversion to the oxidized dimer, GSSG (Shertzer *et al.*, 1995), and marked induction of ceramide production (about 7-fold after 30 min of stimulation) indicating that intracellular level of GSH is the important regulator of degradation of SM to ceramide. Consistent with this, treatment of cells with NAC blocked diamide-mediated decrease in GSH level and induction of ceramide production. Similar to diamide, buthione-(SR)-sulfoximine also decreased the level of GSH and induced the production of ceramide.

The inventor investigated the intracellular level of GSSG in astrocytes treated with TNF- α and diamide. Rat primary astrocytes were incubated with TNF- α (50 ng/ml) and diamide (0.5 mM), and at different time intervals (0, 15, 30, 45, and 60 minutes) the intracellular level of GSSG (100% value is 4.9 ± 0.52 nmol/mg protein) was measured as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. In contrast to the decrease in intracellular level of GSH, both TNF- α and diamide increased the intracellular level of GSSG. Thus it appears that the low GSH and/or high intracellular oxidant (ROS) levels induced by cytokines and thiol-depleting agents facilitated the induction of ceramide production, whereas the normal levels of GSH and/or low ROS induced or maintained by the addition of NAC under these conditions blocked the hydrolysis of sphingomyelin to ceramide. Taken together, these results demonstrate that the intracellular levels of GSH and/or ROS regulate the extent to which sphingomyelin is degraded to ceramide, and ceramide-mediated signaling cascades are transduced.

Aminotriazole and Hydrogen Peroxide Induce the Production of Ceramide in Rat Primary Astrocytes Inhibition of cytokine-mediated induction of ceramide production by antioxidants and induction of ceramide production by thiol-depleting agents alone indicate the possible involvement of ROS in the induction of ceramide production. Therefore, the inventor examined the effect of exogenous addition of an oxidant like H₂O₂ or endogenously produced H₂O₂ by inhibition of catalase with aminotriazole (ATZ), which inhibits endogenous catalase to increase the level of H₂O₂, on the induction of ceramide production. Rat primary astrocytes were incubated with 5 mM aminotriazole (ATZ) or 0.5 mM H₂O₂ in presence or absence of 10 mM NAC. At different time intervals (0, 15, 30, 45, and 60 minutes), cells were washed with HBSS and scraped off. Lipids were extracted, and the level of ceramide (100% value is 4.51 ± 0.1 nmol/mg protein) was measured as described under "Materials and Methods." Results are mean ± S.D. of three different studies. Approximately 45 min following the addition of ATZ, ceramide generation increased more than 5-fold over base line. However, pretreatment of cells with NAC blocked the ATZ-mediated increase in ceramide production. Consistent with the increase in ceramide production by ATZ, addition of exogenous H₂O₂ to astrocytes also induced the production of ceramide with the maximum increase of about 7-fold after 15 min. These results clearly indicate that intracellular levels of ROS regulate the production of ceramide.

Inhibition of Cytokine-mediated Production of Ceramide in Rat Primary Microglia, Oligodendrocytes, and C₆ Glial Cells by NAC Since NAC inhibited the cytokine-mediated production of ceramide in rat primary astrocytes, the inventor examined the effect of NAC on cytokine-mediated induction of ceramide production in rat primary oligodendrocytes, microglia and C₆ glial cells. Rat primary microglia, oligodendrocytes, and C₆ glial cells preincubated with 10 mM NAC for 1 h in serum-free DMEM/F-12 received TNF-α (50 ng/ml). Cells were washed with HBS and scrapped off at different intervals (0, 5, 30, 45, and 60 minutes). Lipids were extracted, and ceramide content (100% value for microglia, oligodendrocytes, and C₆ glial cells are 2.72 ± 0.53, 3.37 ± 0.32, 4.73 ± 0.21 nmol/mg protein, respectively) was measured as described under "Materials and Methods." Results were determined as the mean ± SD. of three different studies. The addition of TNF-α to microglia, oligodendrocytes, or C₆ glial cells induced the production of ceramide. The increase in ceramide in these cells ranges from 2.5- to 4-fold with highest increase in glial cells and lowest in oligodendrocytes. The ceramide levels

peaked in glial cells at 30 min following stimulation and 45 min of stimulation in oligodendrocytes and C₆ glial cells. These observations show that similar to astrocytes, the SM cycle is also present in microglia, oligodendrocytes and C₆ glial cells. Consistent with the effect of NAC on the production of ceramides in astrocytes, this antioxidant also potentially blocked the TNF- α -induced production of ceramide in microglia, oligodendrocytes, and C₆ glial cells indicating that ROS are also involved in cytokine-mediated ceramide production in these cells.

NAC Inhibits TNF- α and Diamide-mediated Apoptosis in Rat Primary Oligodendrocytes by Increasing the Intracellular Level of GSH and Decreasing the Production of Ceramide

The inventor investigated the effect of NAC on TNF- α as well as diamide-mediated apoptosis in rat primary oligodendrocytes as evidenced by electrophoretical detection of hydrolyzed DNA fragment patterns ("laddering"). To understand the role of the intracellular level of GSH in inducing apoptosis, the inventor treated oligodendrocytes with TNF- α or with diamide, a thiol-depleting agent. Rat primary oligodendrocytes preincubated with 10 mM NAC for 1 h received either diamide (0.5 mM) or TNF- α (50 ng/ml). After 12 h of incubation, cells were harvested and washed with phosphate-buffered saline, and genomic DNA was extracted and run on agarose gels as described under "Materials and Methods." Ten micrograms of DNA was loaded in each lane. This study was repeated three times. Levels of ceramide (100% value is 3.37 ± 0.32 nmol/mg protein) and GSH were measured in homogenates as described under "Methods and Materials." Results were measured as the mean \pm S.D. of three different studies. Both TNF- α and diamide decreased the intracellular level of GSH, increased the level of ceramide, and induced internucleosomal DNA fragmentation as evident from the typical ladder pattern. Interestingly, blocking of the diamide- and TNF- α -mediated decrease in intracellular levels of GSH by pretreatment with NAC inhibited the induction of ceramide formation and DNA fragmentation indicating that intracellular levels of GSH may regulate apoptosis in oligodendrocytes through ceramide formation. To prove this further, oligodendrocytes were treated with C₂-ceramide (a cell-permeable ceramide analog) in the presence or absence of NAC. Rat primary oligodendrocytes preincubated with 10 nM NAC for 1 h received C₂-ceramide. After 12 h of incubation, cells were harvested and washed with phosphate-buffered saline, and genomic DNA was extracted and run on agarose gels as described under "Materials and Methods." Ten micrograms of DNA was loaded in each lane.

This study was repeated three times. In contrast to the inhibitory effect of NAC on TNF- α -mediated apoptosis, NAC had no effect on C₂-ceramide-mediated apoptosis in oligodendrocytes.

DNA Fragmentation in Banked Human Brains with X-ALD and MS To understand the underlying relationship among intracellular levels of GSH, levels of ceramide, and DNA fragmentation in cytokine-inflamed central nervous system of X-ALD and MS, the inventor measured the levels of GSH and ceramide in homogenates and also studied the DNA fragmentation in nuclei from brains of patients with X-ALD and MS. Regions surrounding plaques of human brain white matter were used for DNA laddering and to measure the levels of ceramide and GSH. Controls were age- and sex-matched controls for X-ALD and MS, respectively. Since there was no plaque in control brains, the inventor used white matter of control brain for this study. Genomic DNA isolated from nuclei of banked human brains was run on agarose gel and photographed as described under "Materials and Methods." Ten micrograms of DNA was loaded in each lane. This study was reproduced three times. The same amount of brain material (based on protein concentration) was used to measure the level of ceramide as described under "Materials and Methods." Results were measured as the mean \pm S.D. of three different studies. Concentrations of ceramide in the X-ALD and MS controls were 46.6 ± 2.56 and 61.6 ± 6.69 nmol/mg protein, respectively. The concentration of GSH was measured in homogenates as described under "Materials and Methods." Results for this experiment was the mean \pm S.D. of three different studies. In contrast to white matters of control brains, white matters of both X-ALD and MS brains had several plaque regions. In both X-ALD and MS brain homogenates, the level of GSH was lower (55-70% of control), and the level of ceramide was higher (2-3 fold) compared with those found in control brains. Consistent with a lower level of GSH and a higher level of ceramide, genomic DNA isolated from nuclei of X-ALD and MS brains when run on agarose gels formed the typical ladder pattern, an indicator of apoptosis, which was absent in both of the normal brains.

To confirm apoptosis in regions surrounding the plaques of white matters of X-ALD and MS, paraffin-embedded tissue sections of X-ALD and MS were stained with terminal deoxynucleotidyltransferase-mediated fragment end labeling. Terminal deoxynucleotidyltransferase-mediated end labeling of 3'-OH ends of DNA fragments on paraffin-embedded tissue sections (control, X-ALD, and MS) was carried out using a

commercially available kit from Calbiochem. Regions surrounding plaques were used for this study. Consistent with increased DNA fragmentation (apoptosis) in isolated nuclei of X-ALD and MS, the inventor observed increased terminal deoxynucleotidyltransferase staining on brain sections of X-ALD and MS compared with those of controls. These biochemical and morphological observations indicate that intracellular level of GSH may be an important factor in cytokine-mediated degradation of SM to ceramide and apoptosis in inflammatory demyelinating diseases like X-ALD and MS.

Discussion

The inventor shows that intracellular GSH plays a crucial role in the breakdown of SM to ceramide, in that low GSH levels are required for ceramide generation and high GSH levels inhibit production of ceramide. Inhibition of cytokine-mediated breakdown of SM to ceramide by antioxidants like *N*-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) and induction of ceramide production by oxidants or pro-oxidants like hydrogen peroxide, aminotriazole, diamide, and L-buthione-(SR)-sulfoximine clearly delineate a novel function of ROS and GSH in regulation of the first step of sphingomyelin signal transduction pathway. Moreover, decreased levels of GSH and increased levels of ceramide correlate with the DNA fragmentation in rat primary oligodendrocytes as well as in the banked human brains from patients with neuroinflammatory diseases (e.g., multiple sclerosis and X-adrenoleukodystrophy).

The present study underlines the importance of reactive oxygen species in cytokine-mediated degradation of sphingomyelin (SM) to ceramide. Treatment of rat primary astrocytes with tumor necrosis factor- α (TNF- α) or interleukin-1 β led to marked alteration in cellular redox (decrease in intracellular GSH) and rapid degradation of SM to ceramide. Interestingly, pretreatment of astrocytes with *N*-acetylcysteine (NAC), an antioxidant and efficient thiol source for glutathione, prevented cytokine-induced decrease in GSH and degradation of sphingomyelin to ceramide, whereas treatment of astrocytes with diamide, a thiol-depleting agent, alone caused degradation of SM to ceramide. Moreover, potent activation of SM hydrolysis and ceramide generation were observed by direct addition of an oxidant like hydrogen peroxide or a prooxidant like aminotriazole. Similar to NAC, pyrrolidinedithiocarbamate, another antioxidant, was also found to be a potent inhibitor of cytokine-induced degradation of SM to ceramide indicating that cytokine-induced hydrolysis of

sphingomyelin is redox-sensitive. Besides astrocytes, NAC also blocked cytokine-mediated ceramide production in rat primary oligodendrocytes, microglia, and C₆ glial cells. Inhibition of TNF- α - and diamide-mediated depletion of GSH, elevation of ceramide level, and DNA fragmentation (apoptosis) in primary oligodendrocytes by NAC, and observed depletion of GSH, elevation of ceramide level, and apoptosis in banked human brains from patients with neuroinflammatory diseases (*e.g.*, X-adrenoleukodystrophy and multiple sclerosis) indicate that the intracellular level of GSH may play a critical role in the regulation of cytokine-induced generation of ceramide leading to apoptosis of brain cells in these diseases.

Changes in the cellular redox state toward either prooxidant or antioxidant conditions have profound effects on cellular functions. Several lines of evidence presented in this work indicate that the first step of cytokine-induced sphingomyelin signal transduction pathway (*i.e.* breakdown of sphingomyelin to ceramide and phosphocholine) is redox-sensitive. First, cytokines like TNF- α and IL-1 β decreased intracellular GSH and induced the degradation of sphingomyelin to ceramide in rat primary astrocytes, oligodendrocytes, microglia, and rat C₆ glial cells, and pretreatment of the cells with antioxidants like NAC restored the levels of GSH and blocked the degradation of sphingomyelin to ceramide. Second, depletion of endogenous glutathione by diamide or buthione sulfoximine alone induces the degradation of sphingomyelin to ceramide which is blocked by NAC. Third, the increase in intracellular H₂O₂ by the addition of exogenous H₂O₂ or by the inhibition of endogenous catalase by aminotriazole induced the degradation of sphingomyelin to ceramide which is also blocked by NAC. Fourth, besides NAC, PDTC, an antioxidant but not the precursor of GSH (Laight *et al.*, 1997), also inhibited the TNF- α - and IL-1 β -induced hydrolysis of sphingomyelin to ceramide.

Over the years a number of sphingomyelinase activities have been observed in the cell. The major activities are the acid sphingomyelinase present in lysosomes, an enzyme with deficient activity in Niemann-Pick disease (Spence, 1993), and plasma membrane-associated magnesium-dependent neutral pH optimal sphingomyelinase (Chatterjee, 1993). In addition, a cytosolic magnesium-independent (Okazaki *et al.*, 1994) and zinc-dependent acidic (Schissel *et al.*, 1996) sphingomyelinase have also been reported. The lysosomal acidic sphingomyelinase is believed to be responsible for degradation of sphingomyelin associated with turnover of membrane. The membrane-associated neutral sphingomyelinase is known to be activated in serum deprivation, TNF- α , and Fas-associated growth suppression and

apoptosis (Tepper *et al.*, 1995; Weigman *et al.*, 1994). Although the studies reported here do not identify the sphingomyelinase that is redox-sensitive, it is likely that the observed redox-sensitive hydrolysis of sphingomyelin in cytokine-induced production of ceramide is mediated by the plasma membrane-associated neutral sphingomyelinase.

5 The inventor's studies showing DNA fragmentation and increase in ceramide and decrease in GSH in primary oligodendrocytes and banked human brains with X-ALD and MS clearly indicate that intracellular redox (level of GSH) is an important regulator of apoptosis *via* controlling the generation of ceramide. The inventor's conclusion is based on the following observations. First, treatment of oligodendrocytes with TNF- α decreased intracellular level of
10 GSH, increased degradation of SM to ceramide, and induced DNA fragmentation; however, pretreatment of oligodendrocytes with NAC blocked the TNF- α - mediated decrease in GSH level, increase in ceramide level, and increase in DNA fragmentation. In contrast, NAC had no effect on ceramide-mediated DNA fragmentation. Second, treatment of oligodendrocytes only with diamide, a thiol-depleting agent, decreased intracellular level of GSH, increased level of
15 ceramide, and induced DNA fragmentations which are prevented by pretreatment of NAC, a thiol-replenishing agent. Third, the inventor observed increased fragmentation of DNA in the white matter region surrounding plaques from patients with X-ALD and MS where the levels of GSH and ceramide were lower and higher, respectively, compared with those found in white matters of control human brains. These observations indicate that maintenance of the
20 thiol/oxidant balance is crucial for protection against cytokine-mediated ceramide production and thereby against ceramide-induced cytotoxicity.

Observations described herein have demonstrated that ceramide potentiates the cytokine-mediated induction of inducible nitric oxide synthase in astrocytes and C₆ glial cells. Although ceramide by itself did not induce the expression of inducible nitric oxide synthase and
25 production of NO, it markedly stimulated the cytokine-induced expression of inducible nitric oxide synthase and production of NO indicating that sphingomyelin-derived ceramide generation may be an important factor in cytokine-mediated cytotoxicity in neurons and oligodendrocytes in neuroinflammatory diseases. The NAC, which has been used to block the cytokine-induced ceramide production in this study and to inhibit cytokine-mediated induction
30 of inducible nitric oxide synthase, is a nontoxic pharmaceutical drug that enters the cell readily and serves both as a scavenger of ROS and a precursor of GSH, the major intracellular thiol

(Smilkstein *et al.*, 1988). Therefore, the use of reductants such as NAC or other thiol compounds may be beneficial in restoring cellular redox and in inhibition of cytokine-mediated induction of inducible nitric oxide synthase and breakdown of sphingomyelin thus reducing NO-mediated cytotoxicity as well as ceramide-mediated apoptosis in neuroinflammatory diseases.

EXAMPLE 11

Lovastatin and sodium phenylacetate normalize the levels of very long chain fatty acids in skin fibroblasts of X- adrenoleukodystrophy

The inventor has observed that lovastatin and NaPA inhibit the induction of nitric oxide synthase and proinflammatory cytokines (TNF- α , IL-1 β and IL-6) in rat primary astrocytes, microglia and macrophages indicating that these drugs, alone or in combination, may represent a possible avenue of research for therapeutics directed against cytokine- and NO-mediated brain disorders, particularly in demyelinating conditions. Lovastatin and NaPA have already been approved for medication/drug trials for human diseases. In the current work the inventor provides evidence for the therapeutic intervention against pathognomonic accumulation of VLCFA in X-ALD with these drugs.

Materials and methods

Reagents DMEM, bovine calf serum and Hanks' buffered salt solution (HBSS) were from Gibco. [1-¹⁴C]Lignoceric acid was synthesized by treatment of *n*-tricosanoyl bromide with K¹⁴CN as described previously (Hoshi and Kishimoto, 1973).

Enzyme assay for β -oxidation of lignoceric acid The enzyme activity of [1-¹⁴C]lignoceric acid β -oxidation to acetate was measured in intact cells suspended in HBSS. Briefly, the reaction mixture in 0.25 ml of HBSS contained 50-60 μ g of protein and 6 μ M 1-¹⁴C]lignoceric acid. Fatty acids were solubilized with α -cyclodextrin and β -oxidation of [1-¹⁴C]lignoceric acid was carried out as described previously (Singh *et al.*, 1984; Lazo *et al.*, 1988).

Measurement of VLCFA in fibroblasts Fatty acid methyl ester (FAME) was prepared as described previously by Lepage and Roy (1986) with modifications. Fibroblast cells, suspended in HBSS, were disrupted by sonication to form a homogeneous solution. An aliquot (200 μ l) of this solution was transferred to a glass tube and 5 μ g heptacosanoic (27:0) acid was

added as internal standard and lipids were extracted by Folch partition. Fatty acids were transesterified with acetyl chloride (200 μ l) in the presence of methanol and benzene (4:1) for 2 h at 100°C. The solution was cooled down to room temperature followed by addition of 5 ml 6% potassium carbonate solution at ice-cooled temperature. Isolation and purification of FAME were carried out as detailed by Dacremont *et al.* (1995). Purified FAME, suspended in chloroform, was analyzed by gas chromatograph GC-15A attached with chromatopac C-R3A integrator from Shimadzu Corporation.

Preparation of post-nuclear membrane and Western blot analysis The membranes were prepared as described previously (Contreras *et al.*, 1996). Briefly, the post-nuclear fraction was diluted with an ice-cold solution of 0.1 M sodium carbonate, 30 mM iodoacetamide, pH 11.5. After 30 min of incubation at 4°C, the membranes were sedimented by ultracentrifugation. The sedimented membranes were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with antibodies against ALDP as described (Contreras *et al.*, 1996).

RNA isolation and Northern blot analysis Cultured skin fibroblasts were taken out from culture flasks directly by adding Ultraspec-II RNA reagent (Biotecx) and total RNA was isolated according to the manufacturer's protocol. 20 μ g of RNA from each sample was electrophoretically resolved on 1.2% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and crosslinked using UV Stratalinker (Stratagene, La Jolla, CA). Full length ALDP cDNA was kindly provided by Dr. Patrick Aubourg, INSEAM, Hospital Saint-Vincent-de-Paul, Paris, France. ³²P-labeled cDNA probes were prepared according to the instructions provided with Ready-To-Go DNA labeling kit (Pharmacia Biotech). Northern blot analysis was performed essentially as described for Express Hyb Hybridization solution (Clontech, Palo Alto, CA) at 68°C. GAPDH cDNA probe was used as standard for comparing hybridization signals.

Results

Inhibitors of mevalonate pathway stimulate the β -oxidation of lignoceric acid in X-ALD fibroblasts. First, the inventor studied the effect of mevalonate inhibitors (lovastatin, mevastatin and NaPA) on the β -oxidation of lignoceric acid in control human skin fibroblasts. It is apparent from Table 17 that lovastatin, mevastatin and NaPA stimulated the β -oxidation of lignoceric acid in control human skin fibroblasts. Since the β -oxidation of lignoceric acid is

impaired in X-ALD patients, the inventor studied the effect of these compounds on lignoceric acid β -oxidation in cultured skin fibroblasts of X-ALD. Cultured skin X-ALD fibroblasts were incubated in serum-containing DMEM with different concentrations of lovastatin (0, 2, 4, 6, 8, and 10 μ M) or NaPA (0, 1, 2, 3, 4, and 5 mM) in the presence or absence of 2 μ M lovastatin.

5 After every 24 h, medium was replaced with the addition of fresh reagents. Lignoceric acid β -oxidation was measured after 72 h in cell suspension as described above. Values were measured as the mean \pm S.D. of three different studies. Similar to control fibroblasts, these compounds also stimulated lignoceric acid β -oxidation in X-ALD fibroblasts. Both lovastatin and NaPA stimulated lignoceric acid β -oxidation in X-ALD fibroblasts in a dose-dependent
10 manner. The highest dose of lovastatin found to stimulate lignoceric acid β -oxidation (by 70%) was 5 μ M whereas the highest dose of NaPA found to stimulate lignoceric acid β -oxidation (by 40%) was 5 mM. However, a greater degree of stimulation (more than two-fold) was observed by a combination of lovastatin and NaPA even at a dose lower than the one used individually. Higher doses of lovastatin (10-20 μ M) or NaPA (1-20 mM) were cytotoxic to the X-ALD
15 fibroblasts and did not result in further significant stimulation. In the cell fatty acids are oxidized by mitochondrial and peroxisomal β -oxidation enzyme systems. The inventor examined the effect of etomoxir, an inhibitor of mitochondrial β -oxidation, on the β -oxidation of fatty acids (Mannaerts *et al.*, 1979). Etomoxir had no effect on lovastatin- or NaPA-mediated stimulation of lignoceric acid β -oxidation indicating that the observed
20 stimulation of lignoceric acid β -oxidation was a peroxisomal function.

Modulation of cellular content of VLCFA in X-ALD fibroblasts by lovastatin and NaPA

Since mevalonate inhibitors increased β -oxidation of lignoceric acid in control as well as X-ALD fibroblasts, the inventor examined the effect of these compounds on the in situ levels of VLCFA in X-ALD fibroblasts. Cultured skin X-ALD fibroblasts were incubated in
25 serum-containing DMEM with 5 μ M lovastatin, 5 mM NaPA or the combination of 2 μ M lovastatin and 2 mM NaPA for different days (0, 3, 6, 9, 12, and 15 days), and the ratios of $C_{26:0}/C_{22:0}$ (A) and $C_{24:0}/C_{22:0}$ (B) were measured as described. Values were determined as the mean of two different experiments. Treatment of X-ALD fibroblasts with 5 μ M of lovastatin for different time periods (days) resulted in a time-dependent decrease in the ratios of $C_{26:0}/C_{22:0}$
30 and $C_{24:0}/C_{22:0}$. Within 12-15 days of treatment, the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts decreased to the normal level. Similar to lovastatin, NaPA also lowered the

ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts almost to the normal level after 15 days of treatment. However, consistent with the higher degree of stimulation of lignoceric acid β -oxidation by a combination of lovastatin and NaPA, the same combination lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ to normal levels within 7 days. This decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ was also associated with a decrease in the absolute amounts of $C_{24:0}$ and $C_{26:0}$ whereas no significant change was observed in the levels of $C_{22:0}$ (behenic acid).

Normalization of the levels of VLCFA by lovastatin or NaPA in different X-ALD cells with or without deletion of the X-ALD gene. Although the precise function of ALDP, X-ALD gene product, in the metabolism of VLCFA is not known at the present time, however, accumulation of VLCFA in X-ALD cells with loss or mutations in ALDP and their normalization following transfection of cDNA of ALDP indicate a role of ALDP in the metabolism of VLCFA (Cartier *et al.*, 1995). Therefore, the inventor next attempted to examine whether lovastatin or NaPA were able to lower the levels of VLCFA in X-ALD fibroblast cell lines with mutation or deletion of the X-ALD gene. Western blot analysis of post-nuclear membrane fraction of X-ALD skin fibroblasts with antibodies against ALDP and Northern blot analysis of X-ALD skin fibroblasts for ALDP mRNA were carried out as described. ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene. The status of ALDP mRNA or protein and the rate of β -oxidation of lignoceric acid (Table 18) in different X-ALD fibroblasts indicates that ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene. It is apparent from Table 18 that the treatment of X-ALD fibroblasts with lovastatin or NaPA or a combination of these stimulated the β -oxidation of lignoceric acid (55-80%) and normalized the ratios of $C_{26:0}/C_{22:0}$ indicating that these drugs are capable of lowering the level of VLCFA in X-ALD fibroblasts to the normal levels, irrespective of mutation or deletion of the X-ALD gene, the candidate gene for X-ALD.

Table 17
Lovastatin and NaPA stimulate the β -oxidation of lignoceric acid
in control human skin fibroblasts

Treatment	Lignoceric acid β -oxidation (pmol/h/mg protein)
Control	570.2 \pm 52.3
Lovastatin (5 μ M)	945.7 \pm 105.6
Mevastatin (5 μ M)	889.6 \pm 78.4
NaPA (5 mM)	826.2 \pm 87.2

Cells were treated for 72 h in serum-containing DMEM with the listed reagents; β -oxidation, of lignoceric acid was measured as described in Section 2. Medium was replaced after every 24 h with the addition of fresh reagents. Data are mean \pm S.D. of three different studies.

Table 18-A
Effect of lovastatin and NaPA on (A) β -oxidation of lignoceric acid and (B)
the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in cultured skin fibroblasts of X-ALD

Lignoceric acid β -oxidation (pmol/h/mg protein)				
Cell line	Control	Lovastatin	NaPA	Lovastatin+NaPA
A.				
ALDS2	142.7 \pm 15.7	223.5 \pm 24.1	202.5 \pm 17.4	274.6 \pm 30.5
ALDS5	154.2 \pm 14.2	248.2 \pm 26.2	211.5 \pm 22.6	296.2 \pm 25.6
ALDS6	132.4 \pm 15.9	218.3 \pm 19.8	189.7 \pm 21.2	250.1 \pm 28.3
ALDS3	122.3 \pm 11.7	201.3 \pm 22.3	183.2 \pm 17.3	248.6 \pm 29.6
ALDS4	118.5 \pm 12.6	192.8 \pm 20.5	178.9 \pm 18.3	238.7 \pm 21.1

TABLE 18-B
Effect of lovastatin and NaPA on (A) β -oxidation of lignoceric acid and (B) the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in cultured skin fibroblasts of X-ALD

	$C_{26:0}/C_{22:0}$				$C_{24:0}/C_{22:0}$			
	Control	Lovastatin	Lovastatin+NaPA	Control	Lovastatin	Lovastatin+NaPA	Control	Lovastatin
B.								
ALDS2	0.17 ± 0.022	0.049 ± 0.01	0.04 ± 0.008	1.84 ± 0.25	1.25 ± 0.15	1.14 ± 0.15		
ALDS5	0.18 ± 0.025	0.055 ± 0.008	0.04 ± 0.007	1.94 ± 0.29	1.28 ± 0.21	1.18 ± 0.12		
ALDS6	0.22 ± 0.034	0.058 ± 0.01	0.045 ± 0.008	2.01 ± 0.3	1.31 ± 0.18	1.21 ± 0.14		
ALDS3	0.16 ± 0.024	0.045 ± 0.06	0.03 ± 0.005	1.88 ± 0.21	1.26 ± 0.16	1.19 ± 0.25		
ALDS4	0.19 ± 0.028	0.052 ± 0.07	0.036 ± 0.006	1.96 ± 0.23	1.29 ± 0.02	1.22 ± 0.15		

Cells were incubated in serum containing DMEM with 5 μ M lovastatin, 5 mM NaPA or the combination of 2 μ M lovastatin and 2 mM NaPA for 15 days, and the β -oxidation of lignoceric acid (A) and the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ (B) were measured as described in Section 2. Results are mean \pm S.D. of three different studies. ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene.

Discussion

The present study underlines the importance of lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, and the sodium salt of phenylacetic acid (NaPA), an inhibitor of mevalonate pyrophosphate decarboxylase, alone or in combination, in stimulating the β -oxidation of lignoceric acid ($C_{24:0}$) and in normalizing the pathognomonic accumulation of saturated very long chain fatty acids (VLCFA) in cultured skin fibroblasts of X-adrenoleukodystrophy (X-ALD) in which the ALD gene is either mutated or deleted. The detailed mechanism leading to the decrease in the accumulation of VLCFA in X-ALD fibroblasts is not known, but is likely through the stimulation of peroxisomal β -oxidation of VLCFA. In light of the fact that these compounds also inhibit the induction of proinflammatory cytokines and nitric oxide synthase in astrocytes and microglia, the ability of lovastatin and NaPA to normalize the pathognomonic accumulation of VLCFA in skin fibroblasts of X-ALD identify these drugs as possible therapeutics for the neuroinflammatory disease process in X-ALD.

EXAMPLE 12

Lovastatin for X-Linked Adrenoleukodystrophy in Humans

The inventor has shown in animal studies that lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, and sodium phenylacetate, an inhibitor of mevalonate pyrophosphate decarboxylase, inhibit the induction of inducible nitric oxide synthase and proinflammatory cytokines (tumor necrosis factor (α), interleukin-1(β), and interleukin-6) involved in the pathogenesis of neurologic damage in X-linked adrenoleukodystrophy. The inventor has also shown that lovastatin, sodium phenylacetate, and compounds that increase intracellular cyclic AMP and protein kinase A activity normalize the levels of very-long-chain fatty acids in cultured skin fibroblasts from patients with childhood adrenoleukodystrophy and adrenomyeloneuropathy.

To demonstrate lovastatin's effectiveness in treating elevated VLCFAs in human patients, the inventor has treated seven patients from three families with lovastatin for two to six months. The study was approved by the institutional review board at the inventor's medical school, and the patients provided informed consent. The diagnosis was established in each case

by clinical findings and documentation of elevated plasma levels of very-long-chain fatty acids (C26:0) by two different laboratories. Each patient was treated with 20 mg of lovastatin per day for two weeks; the dose was increased to 40 mg per day if no adverse effects were noted. Plasma very-long-chain fatty acids (C26:0) were measured periodically throughout the study.

- 5 Adverse events and compliance were assessed on the basis of the patients' reports and by periodic measurement of plasma total cholesterol, creatine kinase, aspartate aminotransferase, and alanine aminotransferase.

One patient (Patient 4) was withdrawn from the study because of persistent diarrhea and a marked elevation of serum creatine kinase levels. Another (Patient 5) discontinued treatment.

- 10 The inventor's results (Table 19) show that plasma levels of very-long-chain fatty acids (C26:0) declined from their pretreatment values within one month after the initiation of lovastatin therapy in each patient and remained low and within the normal range for up to six months in the five patients who continued the treatment. Their lower post-treatment cholesterol values (Table 19) provide evidence of compliance with therapy. The short duration and small size of
- 15 the study did not allow the inventor to assess whether there was a clinical benefit.

Table 19
Effect of Lovastatin Therapy on Plasma Levels of Very-Long-Chain Fatty Acids in Patients with X-Linked Adrenoleukodystrophy

Patient No.	Age	Age at Onset	Clinical Phenotype*	Plasma Very-Long-Chain Fatty Acids†						Plasma Total Cholesterol				
				Before Treatment		AT 1	AT 2	AT 3	AT 4	AT 5	AT 6	Before Treatment	After Treatment‡	
				micrograms per milliliter										mg/dl
years														
1	42	28	Cerebral AMN	0.8	0.44	0.42	0.36	0.37	0.21	0.21	0.21	109	96	
2	52	35	AMN and Addison's disease§	0.72	0.42	0.41	0.56	0.35	0.17	0.17	0.17	222	158	
3	55	45	AMN	0.97	0.37	0.5	0.3	0.34	0.41	0.41	0.17	236	204	
4	21	14	Adolescent cerebral ALD	1.14	0.5	0.42	----	0.42	----	----	----	165	167	
5	25	----	Presymptomatic AMN¶	0.75	0.43	0.41	0.43	----	----	----	----	140	149	
6	44	----	Heterozygous	0.37	0.24	0.21	0.21	0.22	0.26	0.26	0.16	280	230	
7	70	45	Heterozygous	0.44	0.3	0.34	0.32	0.34	0.41	0.41	0.17	239	183	

*AMN denotes adrenomyeloneuropathy, and ALD adrenoleukodystrophy.

†The value in 50 normal control subjects was 0.24 ± 0.13 μg per milliliter.

‡Post-treatment total cholesterol levels were obtained at six months for the patients who continued treatment (Patients 1, 2, 3, 6, and 7), at four months for Patient 4, and at three months for Patient 5. Patients 4 and 5 discontinued treatment.

5 §Addison's disease is part of the spectrum of ALD.

¶Patient 5 had nerve-conduction abnormalities consistent with AMN but no symptoms when last examined.

||Patients 6 and 7 were women who carried one copy of the mutant X gene. Patient 6 had no symptoms when last examined; patient 7 had mild spasticity and paresthesia of both legs.

These results indicate that lovastatin treatment may represent a simple, safe, and effective way to reduce the accumulated plasma very-long-chain fatty acids in adult patients with X-linked adrenoleukodystrophy.

EXAMPLE 13

5 **Treatment of Humans with Inhibitors of iNOS and Cytokines**

The inhibitors, induction suppressors, induction enhancers, and stimulators or iNOS and/or proinflammatory cytokines of the present invention may be used in the treatment of cells and organisms such as mammals, including rodents and humans. These suppressors may be used to reduce the induction of iNOS and proinflammatory cytokines, reduce the accumulation
10 of VLCFAs, and treat neuroinflammatory diseases such as X-linked adrenoleukodystrophy and multiple sclerosis. As described in Example 12, lovastatin shows effectiveness in reducing the accumulation of very-long-chain fatty acids in human X-ALD patients. Any of the various inhibitors and/or induction suppressors described herein can be used in a human to treat any disease or disorder wherein a undesirable amount of iNOS and/or proinflammatory cytokines is
15 acting to promote tissue damage. Dosages and combinations of inhibitors, suppressors, and/or other pharmaceuticals that may be used can be determined first through an animal model of a particular disease or disorder, and then tested in a human population. Dosages may be optimized on an individual basis, with routine experimentation by those of skill in the art.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be
achieved. All such similar substitutes and modifications apparent to those skilled in the art are

deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

5

Abbas, A. K., *et al.*, *Cellular and Molecular Immunology*, W. B. Saunders Co., Publisher, Philadelphia, Pa., 1991.

Abbondanzo *et al.*, *Breast Cancer Res. Treat.*, 16:182(#151), 1990.

10

Akamatsu, Ohno, Hirota, Kegoshima, Yodoi, Shigesada, "Redox regulation of the DNA binding activity in transcription factor PEBP2. The roles of two conserved cysteine residues," *J. Biol. Chem.* 272:14497-14500, 1997.

Allred *et al.*, *Breast Cancer Res. Treat.*, 16:182(#149), 1990.

15

Aruoma, O.I., *et al.*, "The antioxidant action of N-acetyl cysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid," *Free Rad. Biol. Med.*, 6:593-597, 1989.

Bagasra, Michaels, Zheng, Bobroski, Spitsin, Fu, Tawadros, Koprowski, "Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis," *Proc. Natl. Acad. Sci.*, 92:12041-12045, 1995.

20

Beasley and Brenner, "Role of nitric oxide in hemodialysis hypotension," *Kidney Int.*, 42, Suppl., 38:S96-S100, 1992.

Beckman *et al.* "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide," *Proc. Natl. Acad. Sci. U.S.A.*, 87:1620-1624, 1990.

25

Beg, Ruben, Scheinman, Haskil, Rosen, Baldwin Jr., "I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention," *Genes de Dev.*, 6:1899-1913, 1992.

Begum and Ragolia, "cAMP counter-regulates insulin-mediated protein phosphatase-2A inactivation in rat skeletal muscle cells," *J. Biol. Chem.*, 271:31166-31171, 1996.

30

Berisha *et al.*, "Nitric oxide as a mediator of oxidant lung injury due to paraquat," *Proc. Natl. Acad. Sci. U.S.A.*, 91:744-749, 1994.

- Blenis, "Signal transduction via the MAP kinases: proceed at your own RSK," *Proc. Natl. Acad. Sci. USA*, 90:5889-5892, 1993.
- Bo, Dawson, Wesselingh, Mork, Choi, Kong, Hanley, Trapp, "Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains," *Ann. Neurol.* 36:778-786, 1994.
- 5 Bonfoco, E., *et al.*, "Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate on nitric oxide/superoxide in cortical cell cultures," *Proc. Natl. Acad. Sci. USA*, 92:7162-7166, 1995.
- Boughton-Smith *et al.*, "Nitric oxide synthase activity in ulcerative colitis and Crohn's disease," *Lancet* 342:338-340, 1993.
- 10 Bradford, M., "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.*, 72:248-254, 1976.
- Bredesen, "Neural apoptosis," *Ann. Neurol.*, 38:839-851, 1995.
- 15 Brown *et al.*, *Breast Cancer Res. Treat.*, 16:192(#191), 1990.
- Brugg, Mitchel, Agid, Ruberg, "Ceramide induces apoptosis in cultured mesencephalic neurons," *J. Neurochem.*, 66:733-739, 1996.
- Buisson *et al.*, "The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia," *Br. J. Pharmacol.*, 106:766-767, 1992.
- 20 Bull *et al.*, *J. Invest. Derm.*, 103:435, 1994.
- Busse, R., *et al.*, "Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells," *FEBS Lett.*, 275:87-90, 1990.
- Caira, Pacot, Bardot, Malki, Latruffe, "Transcriptional and post-transcriptional analysis of peroxisomal protein encoding genes from rat treated with an hypolipemic agent, ciprofibrate," *Biochem. Pharmacol.*, 49:611-619, 1995.
- 25 Cartier, N., J. Lopez, P. Moullier, F. Rocchiccioli, M. O. Rolland, P. Jorge, J. Mosser, J. L. Mandel, P. F. Bougneres, O. Danos, and P. Aubourg. "Retroviral-mediated gene transfer corrects very-long-chain fatty acid metabolism in adrenoleukodystrophy fibroblasts," *Proc. Natl. Acad. Sci. USA*. 92:1674-1678, 1995.
- 30 Casey, P.J., *et al.*, "P21 ras is modified by a farnesyl isoprenoid," *Proc. Natl. Acad. Sci. USA*, 86:8323-8327, 1989.

- Castillo, Martinez-Cayuela, Zafra, Garcia-Peregrin, "Effect of phenylalanine derivatives on the main regulatory enzymes of cholesterologenesis," *Mol. Cell. Biochem.*, 105:21-25, 1991.
- Chatterjee, "Neutral sphingomyelinase," *Adv. LipidRes.*, 26:25-47, 1993.
- Chen, Y and Rosazza, J. P. N., "A bacterial nitric oxide synthase from a *Nocardia* species. Biochem Biophys Res Commun," 203:1251-1258, 1994.
- Chiueh *et al.* "Enhanced hydroxyl radical generation by 2'-methyl analog of MPTP: suppression by clorgyline and deprenyl," *Synapse* 11:346-348, 1992.
- Contreras, M., J. Mosser, J. L. Mandel, P. Aubourg, and I. Singh. "The protein coded by the X-adrenoleukodystrophy gene is a peroxisomal integral membrane protein," *FEBS Lett.* 344:211-215, 1994.
- Contreras, M., T. K. Sengupta, F. Sheikh, P. Aubourg, and I. Singh. "Topology of ATP-binding domain of adrenoleukodystrophy gene product in peroxisomes," *Arch. Biochem. Biophys.* 334:369-379, 1996.
- Corbett and McDaniel, "Selective inhibition of inducible nitric oxide synthase by aminoguanidine," *Methods Enzymol.*, 268:398-408, 1996.
- Cornell, N. W. "Rapid fractionation of cell suspensions with the use of brominated hydrocarbons," *Anal. Biochem.* 102:326-331, 1980.
- Cross, Misko, Lin, Hickey, Trotter, Tilton, "Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice," *J. Clin. Invest.*, 93:2684-2690, 1994.
- Dacremont, G., G. Cocquyt, and G. Vincent. "Measurement of very long chain fatty acids, phytanic acid and pristanic acid in plasma and cultured fibroblasts by gas chromatography," *J. Inher. Metab. Dis.* 18 Suppl. 1:76-83, 1995.
- Dawson, Dawson, London, Brecht, Snyder, "Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures," *Proc. Natl. Acad. Sci. USA*, 88:6368-6371, 1991.
- Dbairo, Perry, Gamard, Platt, Poirier, Obeid, Hannun, "Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-alpha: CrmA and Bcl-2 target distinct components in the apoptotic pathway," *J. Exp. Med.*, 185:481-490, 1997.

- Decrement, Cocquyt, Vincent, "Measurement of very long-chain fatty acids, phytanic and pristanic acid in plasma and cultured fibroblasts by gas chromatography," *J. Inher. Metabl. Dis.*, 18(Suppl. 1):76-83, 1995.
- Devary, Gottlieb, Lau, Karin, "Rapid and preferential activation of the c-jun gene during the mammalian UV response," *Mol. Cell. Biol.*, 11:2804-2811, 1991.
- Devlin, J. *et al.*, "Nitric oxide generation. A predictive parameter of acute allograft rejection," *Transplantation*, 58:592-595, 1994.
- Dignam, J.D., *et al.*, "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei," *Nucleic Acids Res.*, 11:1475-1489, 1983.
- Dobashi, Pahan, Chahal, Singh, "Modulation of endogenous antioxidant enzymes by nitric oxide in rat C6 glial cells," *J. Neurochem.*, 68:1896-1903, 1997.
- Drapier, J-C., *et al.*, "Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophages effector cells," *J. Immunol.*, 140:2829-2838, 1988.
- Eberhardt *et al.*, "Molecular cloning of the rat inducible nitric oxide synthase gene promoter," *Biochem. Biophys. Res. Commun.*, 223:752-756, 1996.
- Eisieik & Leijersfam, "The inducible form of nitric oxide synthase (iNOS) in insulin-producing cells," *Diabetes & Metabolism*, 20:116-122, 1994.
- Evans *et al.*, "Differential effects of monoclonal antibodies to tumor necrosis factor alpha and gamma interferon on induction of hepatic nitric oxide synthase in experimental gram-negative sepsis," *Infec. Imm.*, 60:4133-4139, 1992.
- Feinstein, Galea, Cermak, Chugh, Lyandvert, Reis, "Nitric oxide synthase expression in glial cells: suppression by tyrosine kinase inhibitors," *J. Neurochem.*, 62:811-814, 1994b.
- Feinstein, Galea, Roberts, Berquist, Wang, Reis, "Induction of nitric oxide synthase in rat C₆ glial cells," *J. Neurochem.*, 62:315-321, 1994a.
- Fenyk-Melody, Garrison, Brunnert, Weidner, Shen, Shelton, Mudgett, "Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene," *J. Immunol.*, 160:2940-2946, 1998.
- Frohman, In: PCR™ Protocols: A Guide to Methods and Applications, Academic Press, NY, 1990

- Galea, Feinstein, Reis, "Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures," *Proc. Natl. Acad. Sci. USA*, 89:10945-10949, 1992.
- Ganser, Kemer, Brown, Davisson, Kirschner, "A survey of neurological mutant mice. I. Lipid composition of myelinated tissue in known myelin mutants," *Dev. Neurosci.*, 10:99-122, 1988.
- 5 Geller, D.A., *et al.*, "A central role for IL-1 beta in the *in vitro* and *in vivo* regulation of hepatic inducible nitric oxide synthase: IL-1 beta induces hepatic nitric oxide synthesis," *J. Immunol.*, 155:4890-4898, 1995.
- Geller, Lowenstein, Shapiro, Nussler, Di Silvio, Wang, Nakayama, Simmons, Snyder, Billiar, 10 "Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes," *Proc. Natl. Acad. Sci. USA*, 90:3491-3495, 1993.
- Geng, Y., *et al.*, "Tyrosine kinases are involved with the expression of inducible nitric oxide synthase in human articular chondrocytes," *J. Cell. Physiol.*, 163:545-554, 1995.
- Giulian, D., and T. J. Baker. "Characterization of amoeboid microglia isolated from developing 15 mammalian brain," *J. Neurosci.* 6:2163-2178, 1986.
- Goldstein, J.L., *et al.*, "Regulation of the mevalonate pathway," *Nature*, 343:425-430, 1990.
- Goureau *et al.*, "Lipopolysaccharide and cytokines induce a macrophage-type of nitric oxide synthase in bovine retinal pigmented epithelial cells," *Biochem. Biophys. Res. Commun.*, 186:854-859, 1992.
- 20 Graves, L. M., K. E. Bornfeldt, E. W. Raines, B. C. Potts, S. G. Macdonald, R. Ross, and E. G. Krebs. 1993. "Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells," *Proc. Natl. Acad. Sci. USA*. 90:10300-10304.
- Griffith, "Determination of glutathione and glutathione disulfide using glutathione reductase 25 and 2-vinylpyridine," *Anal. Biochem.*, 106:207-212, 1980.
- Guilian and Baker, "Characterization of ameboid microglia isolated from developing mammalian brain," *J. Neurosci.*, 6:2163-2178, 1986.
- Haga *et al.*, "Synthetic Alzheimer amyloid beta/A4 peptides enhance production of complement C3 component by cultured microglial cells," *Brain Res.*, 601:88-94, 1993.
- 30 Hamid *et al.*, "Induction of nitric oxide synthase in asthma," *Lancet*, 342:1510-1513, 1993.

- Hancock, J.F., *et al.*, "Methylation and proteolysis are essential for efficient membrane binding of prenylated p21^{k-ras(B)}," *EMBO J.*, 10:641-646, 1991.
- Hannun and Bell, "Functions of sphingolipids and sphingolipid breakdown products in cellular regulation," *Science*, 243:500-507, 1989.
- 5 Hannun, "Functions of ceramide in coordinating cellular responses to stress," *Science*, 274:1855-1858, 1996.
- Hannun, "The sphingomyelin cycle and the second messenger function of ceramide," *J. Biol. Chem.*, 269:3125-3127, 1994.
- Hardy, S. J., A. Ferranta, A. Poulos, B. S. Robinson, D. W. Johnson, and A. W. Murray.
- 10 "Effect of exogenous fatty acids with greater than 22 carbon atoms (very long chain fatty acids) on superoxide production by human neutrophils," *J. Immunol.* 153:1754-1761, 1994.
- Hashmi, M., W. Stanley, and I. Singh. "Lignoceroyl-CoASH ligase: enzyme defect in fatty acid β -oxidation system in X-linked childhood adrenoleukodystrophy," *FEBS Lett.*
- 15 196:247-250, 1986.
- Henin *et al.*, "Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase," *FASEB J.*, 9:541-546, 1995.
- Henkel, T., *et al.*, "Rapid proteolysis of I κ B- α is necessary for activation of transcription factor NF- κ B," *Nature*, 365:182-185, 1993.
- 20 Her, Lakhani, Zu, "Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation," *Biochem. J.*, 296:25-31, 1993.
- Hibbs *et al.*, "Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy," *J. Clin. Invest.*, 89:867-877, 1992.
- Hooper, Bagsra, Marini, Zborek, Ohnishi, Kean, Champion, Sarker, Bobroski, Farber, Akaike,
- 25 Maeda, Koprosky, "Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: implications for the treatment of multiple sclerosis," *Proc. Natl. Acad. Sci. USA.*, 94:2528-2533, 1997.
- Hooper, Spitsin, Kean, Champion, Dickson, Chaudhry, Koprowski, "Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple
- 30 sclerosis," *Proc. Natl. Acad. Sci. USA*, 95:675-680, 1998.

- Hoshi, M., and Y. Kishimoto. "Synthesis of cerebronic acid from lignoceric acid by rat brain preparation. Some properties and distribution of the hydroxylation system," *J. Biol. Chem.* 248:4123-4130, 1973.
- Hu, S.X., *et al.*, "Differential regulation by cytokines of human astrocyte nitric oxide production," *Glia*, 15:491-494, 1995.
- Ialenti *et al.*, "Modulation of adjuvant arthritis by endogenous nitric oxide," *Br. J. Pharmacol.*, 110:701-706, 1993.
- Issazadeh, Mustafa, Ljungdahl, Hojeberg, Dagerlind, Elde, Olsson, "Interferon- γ , interleukin 4 and transforming growth factor β in experimental autoimmune encephalomyelitis in Lewis rats: dynamics of cellular mRNA expression in the central nervous system and lymphoid cells," *J. Neurosci. Res.*, 40:579-590, 1995.
- Jaffrey, S.R. *et al.*, "Nitric oxide: a neural messenger," *Annu. Rev. Cell Dev. Biol.*, 11:417-440, 1995.
- Jayadev, Lincardic, Hannun, "Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha," *J. Biol. Chem.*, 269:5757-5763, 1994.
- Jelinek, T., *et al.*, "Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation," *Mol. Cell. Biol.*, 16:1027-1034, 1996.
- Joshi *et al.*, "Effect of aminoguanidine on in vivo expression of cytokines and inducible nitric oxide synthase in the lungs of endotoxemic rats," *Res. Commun. Mol. Pathol. Pharmacol. Mar.*, 91:339-346, 1996.
- Kantey, Feinstein, Papa, Hemi, Karasik, "Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1," *J. Biol. Chem.*, 270:23780-23784, 1995.
- Kaurs & Halliwell, "Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients," *FEBS Lett.*, 350:9-12, 1994.
- Kharitonov *et al.*, "Increased nitric oxide in exhaled air of asthmatic patients," *Lancet*, 343:133-135, 1994.
- Kikuchi, A., *et al.*, "The post-translational modification of ras p21 is important for Raf-1 activation," *J. Biol. Chem.*, 269:20054-20059, 1994.

- Kilbourn *et al.*, "NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide," *Proc. Natl. Acad. Sci. U.S.A.*, 87:3629-3632, 1990.
- 5 Klinkert, Kojima, Lesslauer, Rinner, Lassmann, Wekerle, "TNF-alpha receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview," *J. Neuroimmunol.*, 72(2):163-168, 1997.
- Kolb-Bachofen *et al.*, "Epidermal keratinocyte expression of inducible nitric oxide synthase in skin lesions of psoriasis vulgaris," *Lancet*, 344:139 1994.
- 10 Kolesnick and Golde, "The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling," *Cell*, 77:325-328, 1994.
- Koprowski, Zheng, Heber-Katz, Fraser, Rorke, Fu, Hanlon, Dietzscold, "In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases," *Proc. Natl. Acad. Sci.*, 90:3024-3027, 1993.
- 15 Krasemann, E. W., V. Meier, G. C. Korenke, D. H. Hunneman, and F. Hanefeld. "Identification of mutations in the ALD-gene of 20 families with adrenoleukodystrophy/adrenomyeloneuropathy," *Hum. Genet.* 97:194-197, 1996.
- Kroncke *et al.*, "Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation," *Biochem. Biophys. Res. Commun.*, 175:752-758, 1991.
- 20 Kubes, P., *et al.*, "Nitric oxide: an endogenous modulator of leukocyte adhesion," *Proc. Natl. Acad. Sci. USA*, 88:4651-4655, 1991.
- Kwon, Corbett, Rodi, Sullivan, McDaniel, "Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of NF-k β in the signaling mechanism," *Endocrinology*, 136:4790-4795, 1995.
- 25 Lageweg, W., J. E. C. Sykes, M. Lopes-Cardozo, and R. J. A. Wanders. "Oxidation of very long chain fatty acids in rat brain:cerotic acid is -oxidized exclusively in rat brain peroxisomes," *Biochim. Biophys. Acta* 1085:381-384, 1991.
- Laight, Carrier, Anggard, "Investigation of role for oxidant stress in vascular tolerance development to glyceryl trinitrate in vitro," *Br. J. Pharmacol.*, 120:1477-1482, 1997.
- 30 Lander, H.M., *et al.*, "Activation of human peripheral blood mononuclear cells by nitric oxide generating compounds," *J. Immunol.*, 150:1509-1516, 1993.

- Lander, H.M., *et al.*, "p21^{ras} as a common signaling target of reactive free radicals and cellular redox stress," *J. Biol. Chem.*, 270:21195-21198, 1995.
- Latinis and Koretzky, "Fas ligation induces apoptosis and Jun kinase activation independently of CD45 and Lck in human T cells," *Blood*, 87:871-875, 1996.
- 5 Law, Stimmel, Damore, Carter, Clarke, Wall, "Lipopolysaccharide-induced NF- κ B activation in mouse 70Z/3 pre-B lymphocytes is inhibited by mevilonin and 5'-methyladenosine: roles of protein isoprenylation and carboxylation reactions," *Mol. Cell. Biol.*, 12:103-111, 1992.
- Lazo, O., M. Contreras, A. Bhusan, W. Stanley, and I. Singh. "Adrenoleukodystrophy: 10 Impaired oxidation of fatty acids due to peroxisomal lignoceroyl-CoA ligase deficiency," *Arch. Biochem. Biophys.* 270:722-728, 1989.
- Lazo, O., M. Contreras, M. Hashmi, W. Stanley, C. Irazu, and I. Singh. "Peroxisomal lignoceroyl-CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy," *Proc. Natl. Acad. Sci. USA*. 85:7647-7651, 1988.
- 15 Lazo, Singh, Singh, "Postnatal development and isolation of peroxisomes from brain," *J. Neurochem.*, 56:1343-1353 22, 1991.
- Lee *et al.*, "Nitric oxide mediates Fos expression in the spinal cord induced by mechanical noxious stimulation," *NeuroReport*, 3:841-844, 1992.
- Lepage, G. and C. C. Roy. "Direct transesterification of all classes of lipids in one-step 20 reaction," *J. Lipid Res.* 27:114-120, 1986.
- Li, S., *et al.*, "Raf-1 protein kinase activates the NF- κ B transcription factor by dissociating the cytoplasmic NF- κ B-I κ B complex," *Proc. Natl. Acad. Sci. USA*, 90:9247-9251, 1993.
- Ligtenberg, M., S. Kemp, C. O. Sarde, B. M. van Geel, W. J. Kleijer, P. G. Barth, J. L. Mandel, B. A. van Oost, and P. A. Bolhuis. "Spectrum of mutations in the gene encoding the 25 adrenoleukodystrophy protein," *Am. J. Hum. Gen.* 56:44-50, 1995.
- Linardic, Jayadev, Hannun, "Activation of the sphingomyelin cycle by brefeldin A: effects of brefeldin A on differentiation and implications for a role for ceramide in regulation of protein trafficking," *Cell Grow. Different.*, 7:765-774, 1996.
- Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., 30 Singel, D. J., and Stamler, J. S. "A redox-based mechanism for the neuroprotective and

- neurodestructive effects of nitric oxide and related nitroso-compounds," *Nature* 364:626-632, 1993.
- Liu, Hannun, "Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione," *J. Biol. Chem.*, 272:16281-16287, 1997.
- 5 Lo and Cruz, "Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes," *J. Biol. Chem.*, 270:11727-11730, 1995.
- Lowenstein, C.J., *et al.*, "Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme," *Proc. Natl. Acad. Sci. USA*, 89:6711-6715, 1993.
- Lowick *et al.*, "Inducible production of nitric oxide in osteoblast-like cells and in fetal mouse
10 bone explants is associated with suppression of osteoclastic bone resorption," *J. Clin. Invest.*, 93:1465-1472, 1994.
- Lozano, Berra, Munico, Diaz-Meco, Dominguez, Sanz, Moscat, "Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase," *J. Biol. Chem.*, 269:19200-19202, 1994.
- 15 Maimone, Gregory, Amason, Reder, "Cytokine levels in the cerebrospinal fluid and serum of patients with multiple sclerosis," *J. Neuroimmunol.*, 32:67-74, 1991.
- Mandia *et al.*, *Invest Ophthalmol.*, 35:3673-3689, 1994.
- Mannaerts, G. P., L. J. Debeer, J. Thomas, and P. J. De Schepper. "Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from
20 control and clofibrate-treated rats," *J. Biol. Chem.* 254:4585-4595, 1979.
- Manne, Ricca, Brown, Tuomori, Yan, *et al.*, "Ras farnesylation as a target for novel antitumor agent: potent and selective farnesyl diphosphate analog inhibitors of farnesyl transferase," *Drug Dev. Res.*, 34:121-137, 1995.
- Marietta, M. *Trends Biochem. Sci.* 14:488, 1989.
- 25 Marletta, M.A., "Nitric oxide synthase: aspects concerning structure and catalysis," *Cell*, 78:927-930, 1994.
- Martin, O'Brien, Nishioka, McGahon, Mahboubi, Saido, Green, "Proteolysis of fodrin (non-erythroid spectrin) during apoptosis," *J. Biol. Chem.*, 270:6425-6428, 1995.
- McCarthy, K., *et al.*, "Preparation of separate astroglial and oligodendroglial cultures from rat
30 cerebral tissue," *J. Cell Biol.*, 85:890-902, 1980.

- McCartney-Francis, N., *et al.*, "Suppression of arthritis by an inhibitor of nitric oxide synthase," *J. Exp. Med.*, 178:749-754, 1993.
- McGuinness, Powers, Bias, Schmeckpeper, Segal, Gowda, Wesselingh, Berger, Griffin, Smith,
5 "Human leukocyte antigens and cytokine expression in cerebral inflammatory demyelinating lesions of X-linked adrenoleukodystrophy and multiple sclerosis," *J. Neuroimmunol.*, 75:174-182, 1997.
- McGuinness, M. C., D. E. Griffin, G. V. Raymond, C. A. Washington, H. W. Moser, and K. D. Smith. "Tumor necrosis factor- and X-linked adrenoleukodystrophy," *J. Neuroimmunol.* 61:161-169, 1995.
- 10 Mehta, K., *et al.*, "Inhibition by all-trans-retinoic acid of tumor necrosis factor and nitric oxide production by peritoneal macrophages," *J. Leukocyte Biol.*, 55:336-342, 1994.
- Meller *et al.*, "Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex," *Europ. J. Pharmacol.*, 214:93-96, 1992.
- 15 Menon, S.D., *et al.*, "Differential induction of nuclear NF-kB by protein phosphatase inhibitors in primary and transformed human cells," *J. Biol. Chem.*, 268:26804-26812, 1993.
- Merrill and Benveniste, "Cytokines in inflammatory brain lesions: helpful and harmful," *Trends Neurosci.*, 19:331-338, 1996.
- Merrill, J.E., Ignarro, Sherman, Melnick, Lane., "Microglial cell cytotoxicity of
20 oligodendrocytes is mediated through nitric oxide," *J. Immunol.*, 151:2132-2141, 1993.
- Michel, von Echten-Deckert, Rother, Sandoff, Wang, Merrill Jr., "Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide," *J. Biol. Chem.*, 272:22432-22437, 1997.
- Miller *et al.*, "Amelioration of chronic ileitis by nitric oxide synthase inhibition," *J. Pharmacol.*
25 *Exp. Ther.*, 264:11-16, 1993.
- Miller *et al.*, *Lancet*, 34:465-466, 1993.
- Mitrovic, B., Ignarro, Montestruque, Smoll, Merrill, "Nitric oxide as a potential pathological mechanism in demyelination: its differential effects on primary glial cells *in vivo*," *Neurosci.*, 61:575-585, 1994.
- 30 Mittal, C.K., "Nitric oxide synthase: involvement of oxygen radicals in conversion of L-arginine to nitric oxide," *Biochem. Biophys. Res. Commun.*, 193:126-132, 1993.

- Moncada *et al.* "Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication," *Biochem. Pharmacol.*, 38:1709-1715, 1989.
- Moore *et al.*, "7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure," *Brit. J. Pharmacol.*, 108:296-297, 1992.
- Moore *et al.*, "L-NG-nitro arginine methyl ester exhibits antinociceptive activity in the mouse," *Brit. J. Pharmacol.*, 102:198-202, 1991.
- Moser, H. W. "Clinical and therapeutic aspects of adrenoleukodystrophy and adrenomyeloneuropathy," *J. Neuropathol. Expt. Neurol.* 54:740-744, 1995.
- 10 Moser, H. W., A. E. Moser, I. Singh, and B. P. O'Neill. "Adrenoleukodystrophy: survey of 303 cases-biochemistry, diagnosis, and therapy," *Ann. Neurol.* 16:628-641, 1984.
- Moser, Smith, Moser, "X-linked adrenoleukodystrophy. In: *The metabolic and molecular bases of inherited disease*, 7th ed., Scriver OR, Beaudet AL, Sly WS, Valle D, eds., New York: McGraw-Hill, Vol. 2, 232549, 1995.
- 15 Mosser, J., A. M. Douar, C. O. Sarde, P. Kioschis, R. Feil, H. Moser, A. M. Poustka, J. L. Mandel, and P. Aubourg. P. "Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters," *Nature* 361:726-730, 1993.
- Muhl *et al.*, "Expression of nitric oxide synthase in rat glomerular mesangial cells mediated by cyclic AMP," *Br. J. Pharmacol.*, 112:1-8, 1994.
- 20 Mulligan *et al.*, "Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis," *Br. J. Pharmacol.*, 107:1159-1162, 1992.
- Nagafuji *et al.*, "Blockade of nitric oxide formation by N omega-nitro-L-arginine mitigates ischemic brain edema and subsequent cerebral infarction in rats," *Neurosci.*, 147:159-162, 1992.
- 25 Nakamura *et al.*, In: *Enzyme Immunoassays: Heterogeneous and Homogeneous Systems*, Chapter 27, 1987.
- Nathan, C., "Nitric oxide as a secretory product of mammalian cells," *FASEB J.*, 6:3051-3064, 1992.
- Nathan, C.F., "Secretory products of macrophages," *J. Clin. Invest.*, 79:319-326, 1987.

- Nishiya, T., Uehara, Nomura, "Herbimycin A suppresses NF-k β activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells," *FEBS Lett.*, 371:333-336, 1995.
- Okazaki, Bielawska, Domae, Bell, Hannun, "Characteristics and partial purification of a novel
5 cytosolic, magnesium-independent, neutral sphingomyelinase activated in the early signal transduction of 1 α ,25-dihydroxyvitamin D3-induced HL-60 cell differentiation," *J. Biol. Chem.*, 269:4070-4077, 1994.
- Olesen *et al.*, "Nitric oxide is a key molecule in migraine and other vascular headaches," *Trends Pharmacol Sci.*, 15:149-153, 1994.
- 10 Petros *et al.*, "Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock," *Lancet*, 338:1557-1558, 1991.
- Physicians' Desk Reference, Medical Economics Company, Inc., Montvale, N.J., 1998.
- Powers, J. M. "The pathology of peroxisomal disorders with pathogenetic considerations," *J. Neuropathol. Expt. Neurol.* 54:710-719, 1995.
- 15 Powers, J. M., Y. Liu, A. B. Moser, and H. W. Moser. "The inflammatory myelinopathy of adrenoleukodystrophy: cells, effector molecules, and pathogenetic implications," *J. Neuropathol. Expt. Neurol.* 51:630-643, 1992.
- Priess, Loomis, Bishop, Stein, Nidel, Bell, "Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat
20 kidney cells," *J. Biol. Chem.*, 261:8597-8600, 1986.
- Radi, R., *et al.*, "Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide," *J. Biol. Chem.*, 266:4244-4250, 1991.
- Reimann *et al.*, "Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. A putative role for Raf-1 in the induction of the IL-1 beta and the TNF-alpha genes," *J.*
25 *Immunol.*, 153:5740-5749, 1994.
- Remington's Pharmaceutical Sciences" 8th and 15th Editions
- Repko and Maltese, "Post-translational isoprenylation of cellular proteins is altered in response to mevalonate availability," *J. Biol. Chem.*, 264:9945-9952, 1989.
- Rizzo, W. B., P.A. Watkins, M. W. Philips, D. Cranin, B. Campbell, and J. Avigan, J.
30 "Adrenoleukodystrophy: Oleic acid lowers fibroblasts saturated C22-26 fatty acids," *Neurology* 36:357-361, 1986.

- Rizzo, W. B., R. T. Leshne, A. Odone, A. L. Dammann, D. A. Craft, M. E. Jensen, S. S. Jennings, S. Davis, R. Jaitly, and J. A. Sgro. "Dietary erucic acid therapy for X-linked adrenoleukodystrophy," *Neurology* 39:1415-1422, 1989.
- 5 Rogers *et al.*, "Complement activation by beta-amyloid in Alzheimer disease," *Proc. Natl. Acad. Sci. U.S.A.* 89(21):10016-10020, 1992.
- Rossomando, Dent, Sturgill, Marshak, "Mitogen-activated protein kinase kinase 1 (MKK1) is negatively regulated by threonine phosphorylation," *Mol. Cell. Biol.*, 14:1594-1602, 1994.
- 10 Rudick and Ransohoff, "Cytokine secretion by multiple sclerosis monocytes. Relationship to disease activity," *Arch. Neurol.*, 49:265-270, 1992.
- Ruuls, Van der Linden, Sontrop, Huitinga, Dijkstra, "Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors," *Clin. Exp. Immunol.*, 103:467-474, 1996.
- Ruzicka *et al.*, *J. Invest. Derm.*, 103:397, 1994.
- 15 Samid, D., *et al.*, "Phenylacetate: A novel nontoxic inducer of tumor cell differentiation," *Cancer Res.*, 52:1988-1992, 1992.
- Samid, D., *et al.*, "Selective activity of phenylacetate against malignant gliomas: Resemblance to fetal brain damage in phenylketonuria," *Cancer Res.*, 54:891-895, 1994.
- Saran *et al.*, "Reaction of NO with O₂⁻. implications for the action of endothelium-derived relaxing factor (EDRF)," *Free Rad. Res. Commun.* 10:221-226, 1990.
- 20 Schiessel, Schuchman, Williams, Tabas, "Zn²⁺-stimulated sphingomyelinase is secreted by many cell types and is a product of the acid sphingomyelinase gene," *J. Biol. Chem.*, 271:18431-18436, 1996.
- Schilling *et al.*, "A new approach in the treatment of hypotension in human septic shock by NG-monomethyl-L-arginine, an inhibitor of the nitric oxide synthetase," *Intensive Care Med.*, 19:227-231, 1993.
- 25 Schmidt, Traenckner, Meier, Baeuerle, "Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-kappa B," *J. Biol. Chem.*, 270:27136-27142, 1995.
- 30 Schreck, R., *et al.*, "Nuclear factor kB: An oxidative stress responsive transcription factor of eukaryotic cells," *Free Rad. Res. Commun.*, 17:221-237, 1992.

- Schreck, Rieber, Baeuerle, "Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1," *EMBO J.*, 10:2247-2258, 1991.
- Sen and Packer, "Antioxidant and redox regulation of gene transcription. *FASEB J.*, 10:709-720, 1996. Antioxidant and redox regulation of gene transcription," *FASEB J.* May;10(7):709-720, 1996.
- Shafer and Murphy, "Activated astrocytes induce nitric oxide synthase-2 in cerebral endothelium *via* tumor necrosis factor- α ," *Glia*, 21:370-379, 1997.
- Sharif, S.F., *et al.*, "Human astrocyte production of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 following exposure to lipopolysaccharide endotoxin," *Neurological Res.*, 15:109-112, 1993.
- Shertzer, Vasilion, Liu, Tabor, Nebert, "Enzyme induction by L-buthionine (S,R)-sulfoximine in cultured mouse hepatoma cells," *Chem. Res. Toxicol.*, 8:431-436, 1995.
- Shin, Drysdale, Shin, Noble, Fisher, Paznekas, "Definition of a lipopolysaccharide-responsive element in the 5'-flanking regions of MuRantes and *crg-2*," *Mol. Cell Biol.*, 14:2914-2925, 1994.
- Sinensky, Beck, Leonard, Evans, "Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis," *J. Biol. Chem.*, 265:19937-19941, 1991.
- Singh, I. "Biochemistry of peroxisomes in health and disease," *Mol. Cell. Biochem.* 167:1-29, 1997.
- Singh, I., A. E. Moser, S. Goldfischer, and H. W. Moser. "Lignoceric acid is oxidized in peroxisome: Implication for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy," *Proc. Natl. Acad. Sci. USA* 81:4203-4207, 1984.
- Singh, I., *et al.*, "Acyl-CoA ligases from rat brain microsomes. An immunochemical study," *Biochim. Biophys. Acta.*, 963:509-514, 1988.
- Smilkstein, M.J., *et al.*, "Efficacy of oral N-acetyl cystein in the treatment of acetaminophen overdose. Analysis of the national multicenter study," *N. Engl. J. Med.*, 319:1557-1562, 1988.
- Sommer, N., P.A. Loschmann, G. H. Northoff, M. Weller, A. Steinbrecher, J. P. Steinbach, R. Lichtenfels, R. Meyermann, A. Riethmuller, A. Fontana, J. Dichgans, and R. Martin.

"The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis," *Nature Med.* 1:244-248, 1995.

Spence, *Adv. Lipid Res.*, "Sphingomyelinases," 26:3-23, 1993.

5 Stadler *et al.*, "Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide," *J. Immunol.*, 147:3915-3920, 1991.

Stefanova, I., Corcoran, Horak, Wahl, Bolen, Horak, "Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn," *J. Biol. Chem.*, 268:20725-20728, 1993.

Stevanovic-Racic *et al.*, *Arth. & Rheum.*, 37, 1062-1069, 1994.

10 Stites, D. P., and Terr, A. I., *Basic and Clinical Immunology*, 7th Ed., Appleton & Lange, Publisher, Norwalk, Conn., 1991.

Stuehr, D.J., *et al.*, "Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells," *J. Expt. Med.*, 169:1543-1555, 1989.

15 Sun, Maggirwar, Harhaj, "Molecular cloning of mouse tissue inhibitor of metalloproteinases-3 and its promoter. Specific lack of expression in neoplastic JB6 cells may reflect altered gene methylation," *J. Biol. Chem.*, 270:18347-18351, 1995.

Suzuki, Mizuno, Packer, "Signal transduction for nuclear factor-kappa B activation. Proposed location of antioxidant-inhibitable step," *J. Immunol.*, 153:5008-5015, 1994.

20 Taupin, Renno, Bourbonniere, Peterson, Rodriguez, Owens, "Increased severity of experimental autoimmune encephalomyelitis, chronic macrophage/microglial reactivity, and demyelination in transgenic mice producing tumor necrosis factor- α in the central nervous system," *Eur. J. Immunol.*, 27:905-913, 1997.

25 Tepper, Jaydev, Liu, Bielawska, Wolff, Yonchara, Hannun, Seldin, "Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity," *Proc. Natl. Acad. Sci. U.S.A.*, 92:8443-8447, 1995.

The Merck Index, 11th Edition, Susan Budavari, Ed., Merck & Co., Inc., Rahway, N.J. U.S.A., 1989.

30 Thibault, Samid, Cooper, Figg, Tompkins, Patronas, Headlee, Kohler, Venzon, Myers, "Phase I study of phenylacetate administration twice daily to patients with cancer," *Cancer*, 75:2932-2938, 1995.

Thiemermann & Vane, *Eur. J. Pharmacol.*, 211:172-182, 1992.

- Thompson, "Apoptosis in the pathogenesis and treatment of disease," *Science*, 267:1456-1462, 1995.
- Tiku, Liesch, Robertson, "Production of hydrogen peroxide by rabbit articular chondrocytes. Enhancement by cytokines," *J. Immunol.*, 145:690-696, 1990.
- 5 Trifiletti *et al.*, "Neuroprotective effects of NG-nitro-L-arginine in focal stroke in the 7-day old rat," *Europ. J. Pharmacol.*, 218:197-198, 1992.
- Tsuji, S., T. Ohno, T. Miyatake, A. Suzuki, and T. Yamakawa. "Fatty acid elongation activity in fibroblasts from patients with adrenoleukodystrophy (ALD)," *J. Biochem.* 96:1241-1247, 1984.
- 10 Tsukada, Miyagi, Matsuda, Yanagisawa, Yone, "Tumor necrosis factor and interleukin-1 in the CSF and sera of patients with multiple sclerosis," *J. Neurol. Sci.*, 104:230-234, 1991.
- van der Veen, Hinton, Incardonna, Hofman, "Extensive 14 peroxynitrite activity during progressive stages of central nervous system inflammation," *J. Neuroimmunol.*, 77:1-7, 1997.
- 15 Verheij, Bose, Lin, Yao, Jarvis, Grant, *et al.*, "Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis," *Nature*, 380:75-79, 1996.
- Villarroya, Violleau, Younes-Chennoufi, Baumann, "Myelin-induced experimental allergic encephalomyelitis in Lewis rats: tumor necrosis factor- α levels in serum and cerebrospinal fluid immunohistochemical expression in glial cells and macrophages of
- 20 optic nerve and spinal cord," *J. Neuroimmunol.*, 64:55-61, 1996.
- Wang, M-H., *et al.*, "Macrophage stimulating protein inhibits induction of nitric oxide production by endotoxin or cytokine-stimulated mouse macrophages," *J. Biol. Chem.*, 269:14027-14031, 1995.
- Watkins, P. A., S. J. Gould, M. A. Smith, L. T. Braiterman, H. M. Wei, F. Kok, A. B. Moser,
- 25 H. W. Moser, and K. D. Smith. "Altered expression of ALDP in X-linked adrenoleukodystrophy," *Am. J. Hum. Genet.* 57:292-301, 1995.
- Weigman, Schutze, Machleidt, Witte, Kronke, "Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling," *Cell*, 78:1005-1015, 1994.
- Welsh *et al.*, "Interleukin-1 beta-induced nitric oxide production in isolated rat pancreatic islets
- 30 requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase," *Endocrinol.*, 129:3167-3173, 1991.

- Welsh, "Interleukin-1 beta-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F," *J. Biol. Chem.*, 271:8307-8312, 1996.
- White *et al.*, "Superoxide and peroxynitrite in atherosclerosis,"
5 *Proc. Natl. Acad. Sci. U.S.A.*, 91:1044-1048, 1994.
- Wiesner and Dawson, "Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway," *J. Neurochem.* 66:1418-1425, 1996.
- Wink, Kasprzak, Maragos, Elespuru, Misra, Dunams, Cebula, Koch., "DNA deaminating ability and genotoxicity of nitric oxide and its progenitors," *Science*, 254:1001-1003,
10 1991.
- Winlaw *et al.*, "Increased nitric oxide production in heart failure," *Lancet*, 344:373-374, 1994.
- Xie, Q-W., Kashiwabara, Nathan., "Role of transcription factor NF-k β /Rel in induction of nitric oxide synthase," *J. Biol. Chem.*, 269:4705-4708, 1994.
- Xie, Q-W., *et al.*, "Promoter of the mouse gene encoding calcium-independent nitric oxide
15 synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharides," *J. Expt. Med.*, 177:1779-1784, 1993.
- Yao, Zhang, Delikat, Mathias, Basu, Kolesnick, "Phosphorylation of Raf by ceramide-activated protein kinase," *Nature*, 378:307-310, 1995.
- Zang, X., *et al.*, "Lipopolysaccharide-induced selective priming effects on tumor necrosis factor
20 alpha and nitric oxide production in mouse peritoneal macrophages," *J. Exp. Med.*, 177:511-516, 1993.
- Zembowicz & Vane, "Induction of nitric oxide synthase activity by toxic shock syndrome toxin 1 in a macrophage-monocyte cell line," *Proc. Natl. Acad. Sci. U.S.A.*, 89:2051-2055, 1992.
- 25 Zhang and Kolesnick, "Signaling through the sphingomyelin pathway," *Endocrinology*, 136:4157-4160, 1995.
- Zhang, Alter, Reed, Bomer, Obeid, Hannun, "Bcl-2 interrupts the ceramide-mediated pathway of cell death," *Proc. Natl. Acad. Sci. U.S.A.*, 93:5325-5328, 1996.

CLAIMS:

1. A method for suppressing the induction of inducible nitric oxide synthase in a cell comprising contacting said cell with an effective amount of at least one induction suppressor of inducible nitric oxide synthase or a proinflammatory cytokine, wherein said induction
5 suppressor is selected from the group consisting of an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an inhibitor of the farnesylation of Ras, an inhibitor of NF-k β activation, an antioxidant, an enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of AMP-activated protein kinase.
- 10 2. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP and (S)-cAMP.
- 15 3. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of mevalonate synthesis.
4. The method of claim 3, wherein said inhibitor of mevalonate synthesis is an inhibitor of HMG-CoA reductase.
- 20 5. The method of claim 4, wherein said inhibitor of HMG-CoA reductase is a stimulator of AMP-activated protein kinase.
6. The method of claim 4, wherein said inhibitor of HMG-CoA reductase is lovastatin or
25 AICAR.
7. The method of claim 1, wherein said inhibitor of inducible nitric oxide synthase is an inhibitor of mevalonate pyrophosphate decarboxylase.
- 30 8. The method of claim 7, wherein said inhibitor of mevalonate pyrophosphate decarboxylase is phenylacetic acid or 4-phenylbutyrate.

9. The method of claim 3, wherein said inhibitor of mevalonate synthesis is lovastatin, mevastatin or AICAR.
- 5 10. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of farnesyl pyrophosphate, and wherein said inhibitor of farnesyl pyrophosphate is 4-phenylbutyrate or NaPA.
11. The method of claim 1, wherein said induction suppressor of inducible nitric oxide
10 synthase is an inhibitor of the farnesylation of Ras.
- 12 The method of claim 11, wherein said inhibitor of the farnesylation of Ras is FPT inhibitor II.
- 15 13. The method of claim 1, wherein said one induction suppressor of inducible nitric oxide synthase is an antioxidant.
14. The method of claim 13, wherein said antioxidant is N-acetyl cysteine or PDTC.
- 20 15. The method of claim 1, wherein said one induction suppressor of inducible nitric oxide synthase is an enhancer of intracellular cAMP.
16. The method of claim 15, wherein said enhancer of intracellular cAMP is forskolin, rolipram, 8-bromo-cAMP or cAMP.
- 25 17. The method of claim 15, wherein said enhancer of intracellular cAMP is an inhibitor of cAMP phosphodiesterase.
18. The method of claim 15, wherein inhibitor of cAMP phosphodiesterase is rolipram,
30 theophylline, papaverine.

19. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is an enhancer of protein kinase A.

20. The method of claim 19, wherein said enhancer of protein kinase A is forskolin,
5 8-Br-cAMP, cAMP or (S)-cAMP.

21. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of NF-k β activation.

10 22. The method of claim 21, wherein said inhibitor of NF-k β activation is AICAR, lovastatin, mevastatin, 4-phenylbutyrate, NaPA or a derivative thereof.

23. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of Ras/Raf/MAP kinase pathway.

15

24. The method of claim 1, wherein said inhibitor of Ras/Raf/MAP kinase pathway is AICAR or a derivative thereof.

25. The method of claim 1, wherein said induction suppressor of inducible nitric oxide
20 synthase is lovastatin or a derivative thereof.

26. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is mevastatin or a derivative thereof.

25 27. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is phenylacetic acid or a derivative thereof.

28. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is N-acetyl cysteine or a derivative thereof.

30

29. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is PDTC or a derivative thereof.

30. The method of claim 1, wherein said induction suppressor of inducible nitric oxide
5 synthase is forskolin or a derivative thereof.

31. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is cAMP or a derivative thereof.

10 32. The method of claim 31, wherein said cAMP derivative is 8-bromo-cAMP or (S)-cAMP.

33. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is FPT inhibitor II or a derivative thereof.

15

34. The method of claim 1, further comprising suppressing the induction of a proinflammatory cytokine.

35. The method of claim 33, wherein said proinflammatory cytokine is selected from the
20 group consisting of TNF- α , IL-1 β , IL-6, IL-2, IL-8 and IFN- γ .

36. The method of claim 1, wherein said induction suppressor of inducible nitric oxide synthase is formulated in a pharmaceutically acceptable vehicle.

25 37. The method of claim 1, wherein said therapeutic dose is administered to a mammal.

38. The method of claim 36, wherein said mammal is a human.

39. A method for suppressing the induction of inducible nitric oxide synthase in a cell
30 comprising contacting said cell with an effective amount of at least one induction suppressor of inducible nitric oxide synthase, wherein said induction suppressor of inducible nitric oxide

synthase is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP and (S)-cAMP.

5

40. A method of identifying a candidate induction suppressor of inducible nitric oxide synthase inhibitor, comprising preparing a cell capable of inducible nitric oxide synthase activity and testing said candidate induction suppressor for the ability to inhibit said inducible nitric oxide synthase activity, wherein said candidate induction suppressor of inducible nitric oxide synthase is an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an inhibitor of the farnesylation of Ras, an inhibitor of NF-k β activation, an antioxidant, an enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of AMP-activated protein kinase, and wherein said ability to inhibit said inducible nitric oxide synthase activity is indicative of a candidate induction suppressor of inducible nitric oxide synthase.

15

41. The method of claim 40, comprising the steps of :

- a) obtaining a cell comprising the capability of inducible nitric oxide synthase activity;
- 20 b) obtaining a candidate induction suppressor of inducible nitric oxide synthase;
- c) admixing said cell with said candidate induction suppressor of inducible nitric oxide synthase; and
- d) determining the ability of said candidate induction suppressor of inducible nitric oxide synthase to inhibit the formation of nitric oxide in the presence of inducible nitric oxide synthase, wherein said inhibition of the formation of nitric oxide in the presence of inducible nitric oxide synthase is indicative of a candidate induction suppressor of inducible nitric oxide synthase.

25

42. The method of claim 41, further comprising measuring inducible nitric oxide synthase gene product.

30

43. The method of claim 42, wherein said inducible nitric oxide synthase gene product is a mRNA message or a protein product.

44. The method of claim 41, further comprising the step of purifying the candidate
5 induction suppressor of inducible nitric oxide synthase.

45. The method of claim 44, wherein said purified candidate induction suppressor of inducible nitric oxide synthase is formulated in a pharmaceutically acceptable vehicle.

10 46. A method of inhibiting nitric oxide cytotoxicity comprising contacting a cell capable of producing nitric oxide with a biologically effective amount of an inducible nitric oxide synthase induction suppressor identified by claim 40.

47. The method of claim 46, wherein said purified candidate induction suppressor of
15 inducible nitric oxide synthase is formulated in a pharmaceutically acceptable vehicle.

48. The method of claim 46, wherein said cell is in a mammal.

49. The method of claim 48, wherein said mammal is a human.
20

50. The method of claim 48, wherein said mammal is a rodent.

51. A method of suppressing the accumulation of very long chain fatty acids in a cell, comprising administering a biologically effective amount of at least one induction suppressor of
25 inducible nitric oxide synthase.

52. The method of claim 51, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an inhibitor of the farnesylation of Ras, an inhibitor of NF- κ B activation, an antioxidant, an
30 enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of AMP-activated protein kinase.

53. The method of claim 51, wherein said induction suppressor of inducible nitric oxide synthase is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA),
5 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP and (S)-cAMP.
54. The method of claim 51, wherein lignoceric acid β -oxidation is stimulated.
- 10 55. The method of claim 54, wherein the amount of very long chain fatty acids in a cell is suppressed.
56. The method of claim 51, wherein ratios of $C_{26:0}/C_{22:0}$ or $C_{24:0}/C_{22:0}$ fatty acids are lowered.
- 15 57. A method of treating a nitric oxide or cytokine mediated disorder in a cell, comprising administering a biologically effective amount of at least one induction suppressor of inducible nitric oxide synthase.
58. The method of claim 57, wherein said induction suppressor of inducible nitric oxide
20 synthase is an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an inhibitor of the farnesylation of Ras, an inhibitor of NF- κ B activation, an antioxidant, an enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of AMP-activated protein kinase.
- 25 59. The method of claim 57, wherein said induction suppressor of inducible nitric oxide synthase is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP and (S)-cAMP.

60. The method of claim 57, wherein said of treating a nitric oxide or cytokine mediated disorder in a cell is a method of treating a nitric oxide mediated disorder.

61. The method of claim 57, wherein said of treating a nitric oxide or cytokine mediated disorder in a cell is a method of treating a cytokine mediated disorder.

62. The method of claim 57, wherein said induction suppressor of inducible nitric oxide synthase is an induction suppressor of at least one proinflammatory cytokine.

63. The method of claim 62, wherein said at least one proinflammatory cytokine is TNF- α , IL-1 β , IL-2, IL-6, IL-8 or IFN- γ .

64. The method of claim 57, wherein said nitric oxide or cytokine mediated disorder is selected from the group consisting of myelinolytic inflammation, a demyelinating condition or an inflammatory demyelinating disease.

65. The method of claim 64, wherein said inflammatory disease is X-ALD, multiple sclerosis or Alzheimer's disease.

66. A method for treatment of septic shock, comprising administering a biologically effective amount of at least one induction suppressor of inducible nitric oxide synthase.

67. The method of claim 66, wherein said induction suppressor of inducible nitric oxide synthase is an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an inhibitor of the farnesylation of Ras, an inhibitor of NF-k β activation, an antioxidant, an enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of AMP-activated protein kinase.

68. The method of claim 66, wherein said induction suppressor of inducible nitric oxide synthase is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA),

5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP and (S)-cAMP.

69. A method for inhibiting apoptosis of a cell comprising contacting said cell with an
5 effective amount of at least one induction suppressor of inducible nitric oxide synthase.

70. The method of claim 69, wherein said induction suppressor of inducible nitric oxide
synthase is an inhibitor of mevalonate synthesis, an inhibitor of Ras/Raf/MAP kinase pathway, an
inhibitor of the farnesylation of Ras, an inhibitor of NF- κ B activation, an antioxidant, an
10 enhancer of intracellular cAMP, an enhancer of protein kinase A (PKA) or a stimulator of
AMP-activated protein kinase.

71. The method of claim 69, wherein said induction suppressor of inducible nitric oxide
synthase is selected from the group consisting of lovastatin, mevastatin, FPT inhibitor II,
15 forskolin, rolipram, phenylacetate, N-acetyl cysteine, PDTC, 4-phenylbutyrate (4PBA),
5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP,
8-bromo-cAMP and (S)-cAMP.

72. A method for enhancing the production of an inducible nitric oxide synthetase or a
20 proinflammatory cytokine in a cell comprising providing a biologically effective
amount of a inducible nitric oxide synthetase stimulator, wherein said stimulator is a PKA
inhibitor or enhancer of intracellular cAMP.

73. The method of claim 72, wherein said PKA inhibitor is H-89, myristoylated PKI or
25 (R)-cAMP.

74. The method of claim 72, wherein said enhancer of intracellular cAMP is forskolin,
8-bromo-cAMP, and rolipram.

30 75. The method of claim 72, wherein said enhancer of intracellular cAMP is an inhibitor of
cAMP phosphodiesterase.

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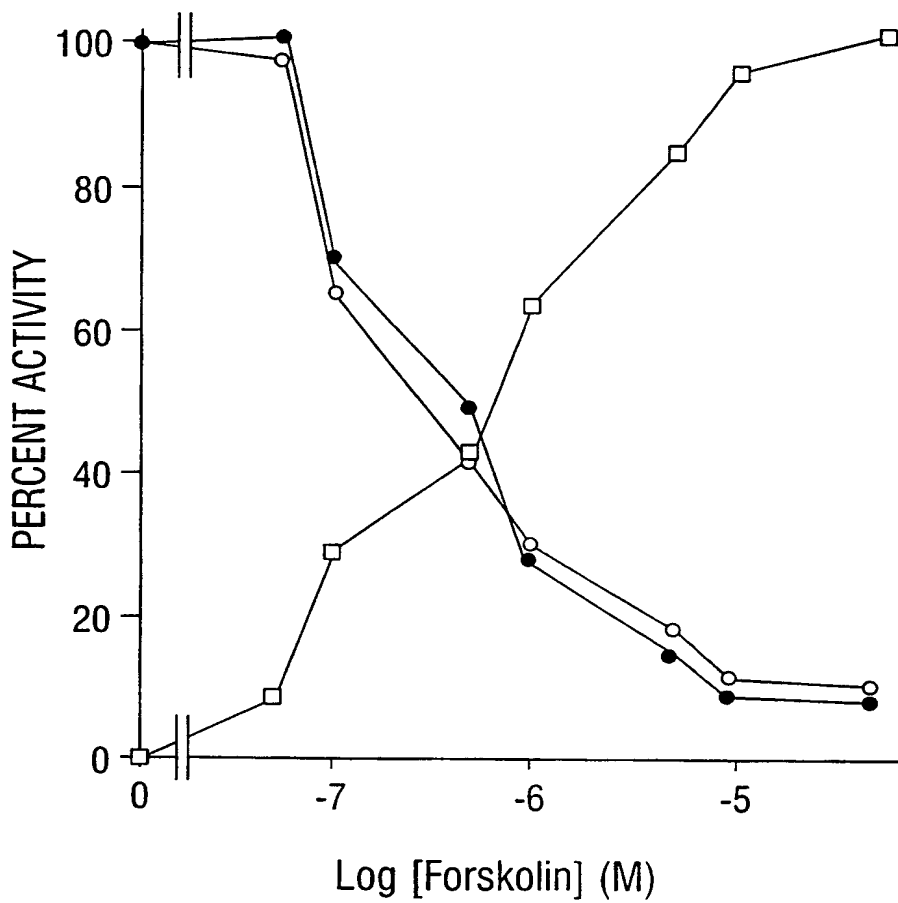
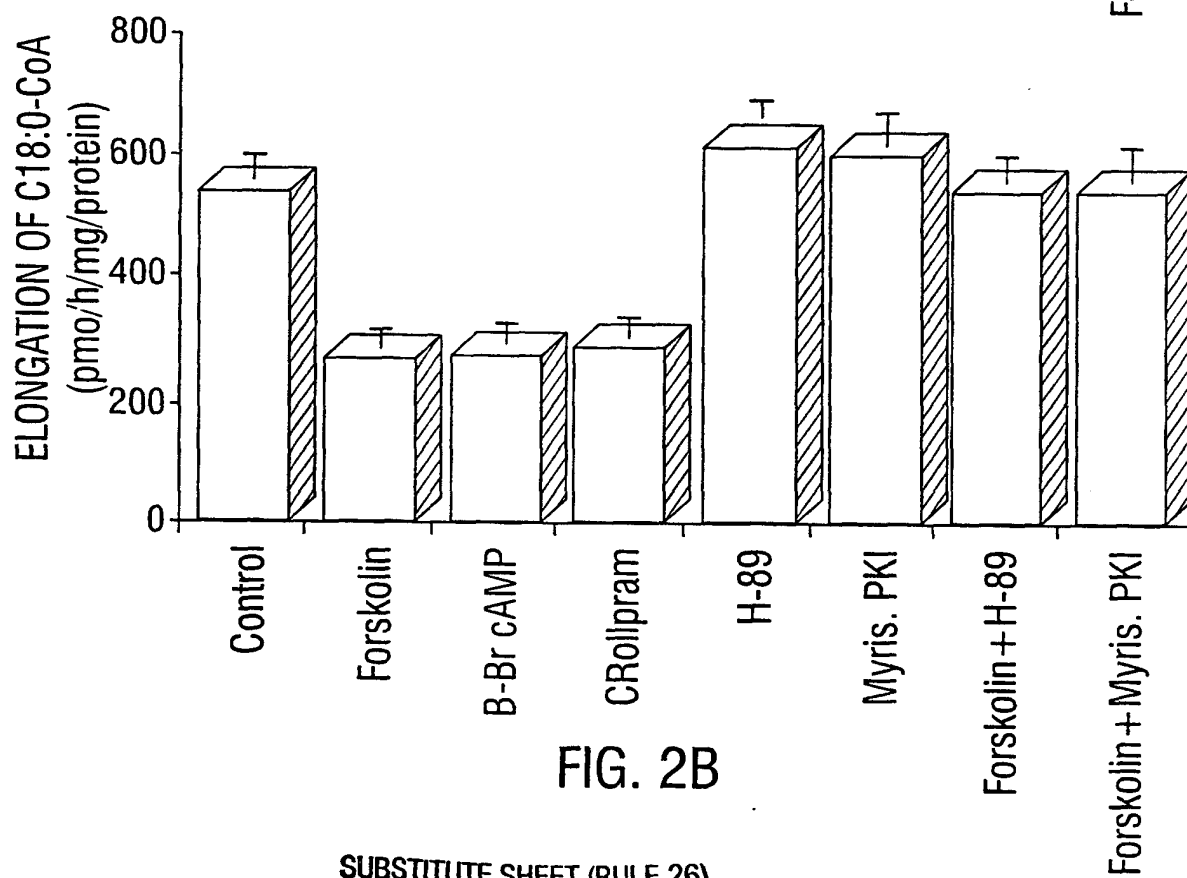
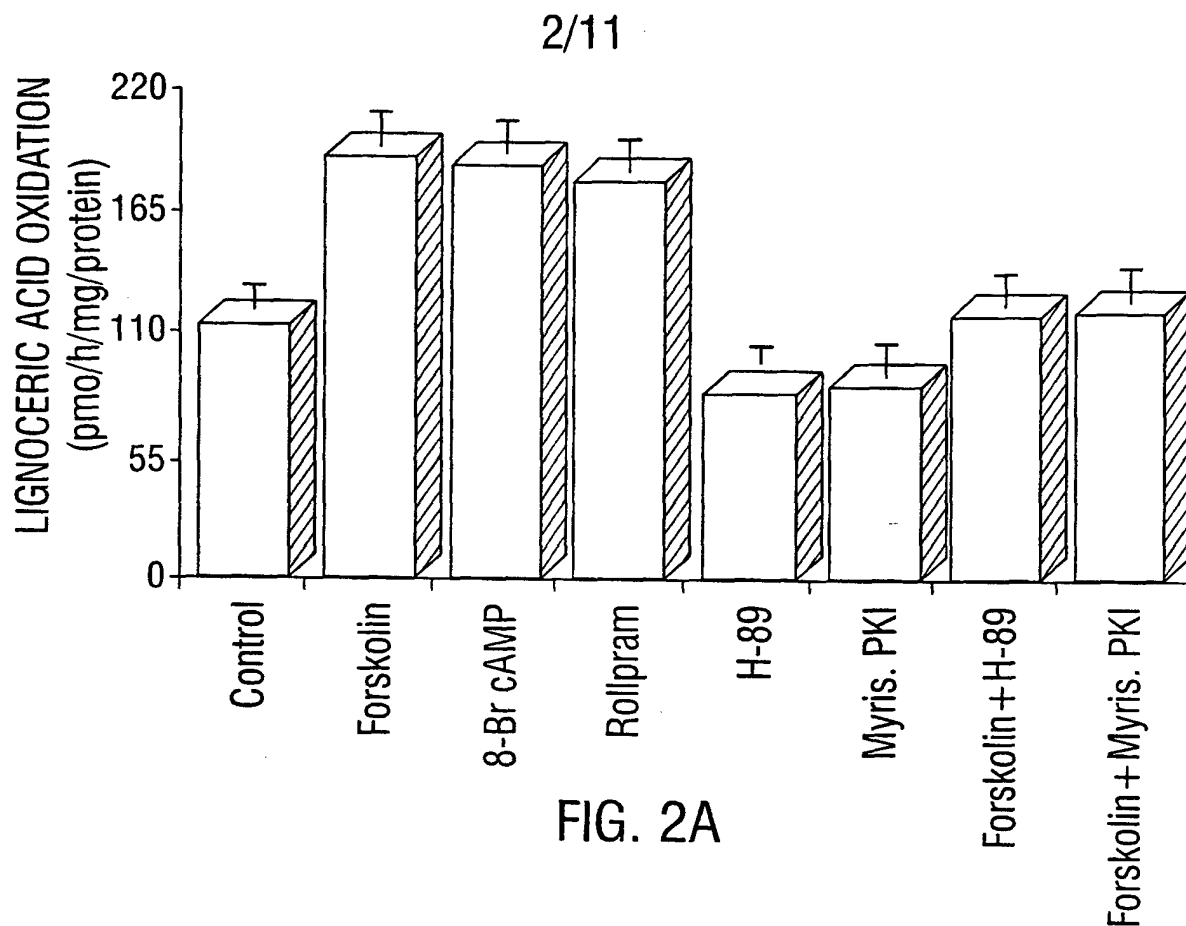


FIG. 1

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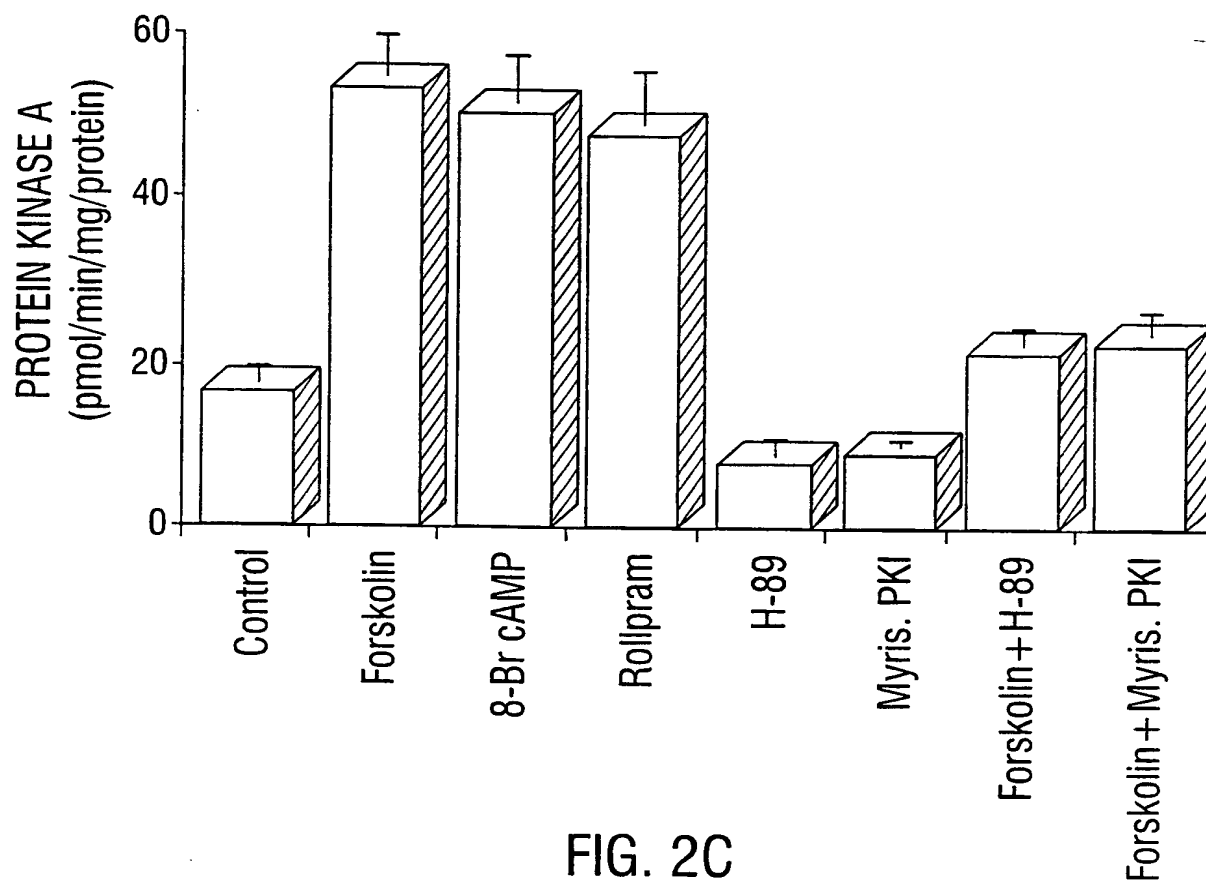


FIG. 2C

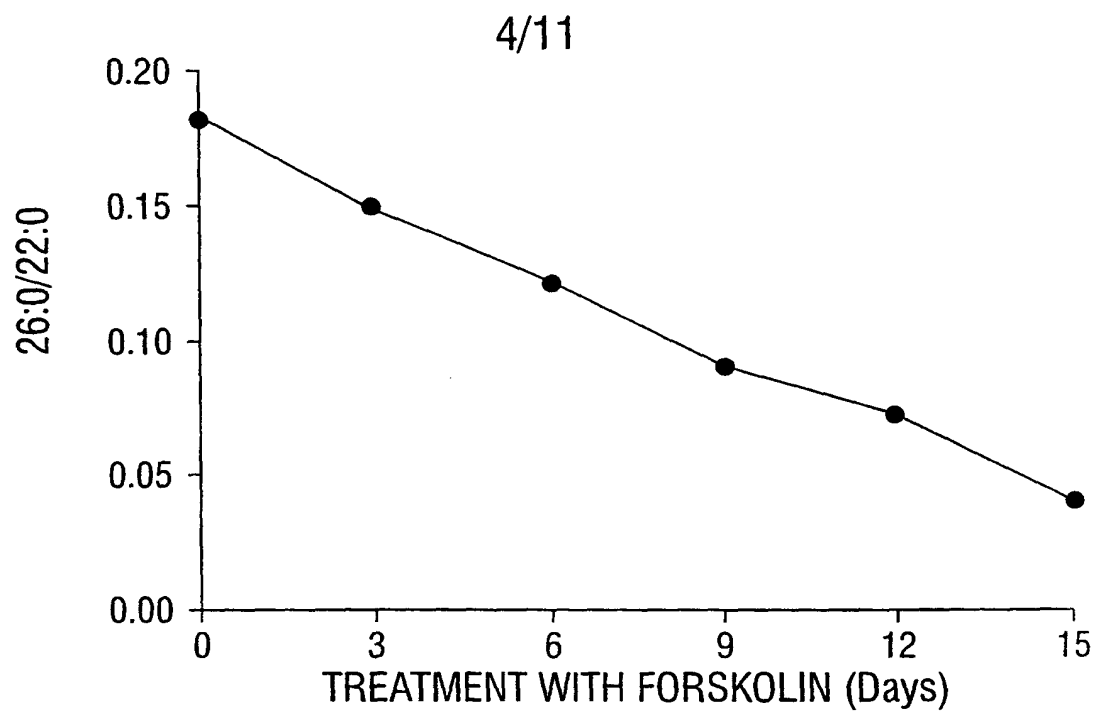


FIG. 3A

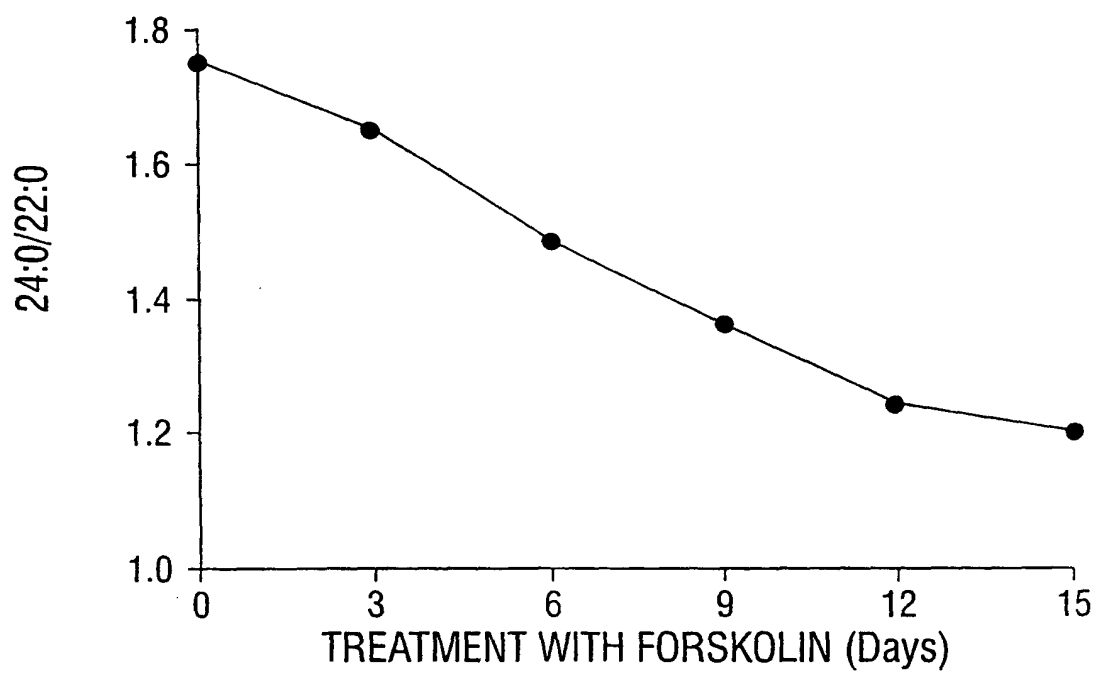


FIG. 3B

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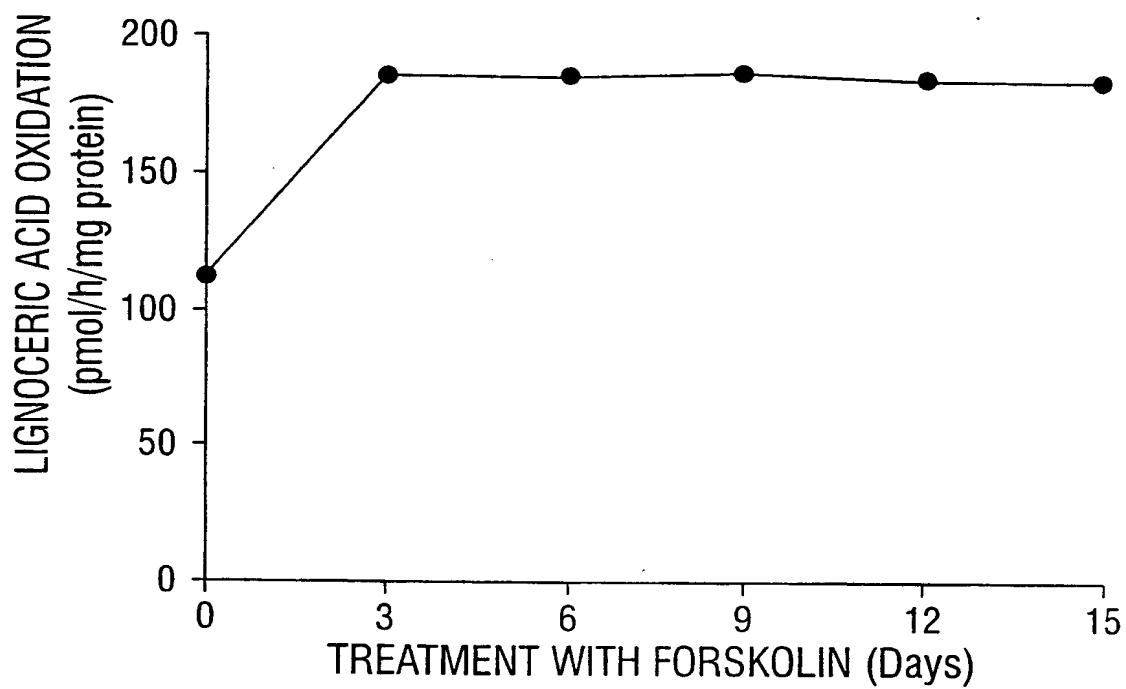
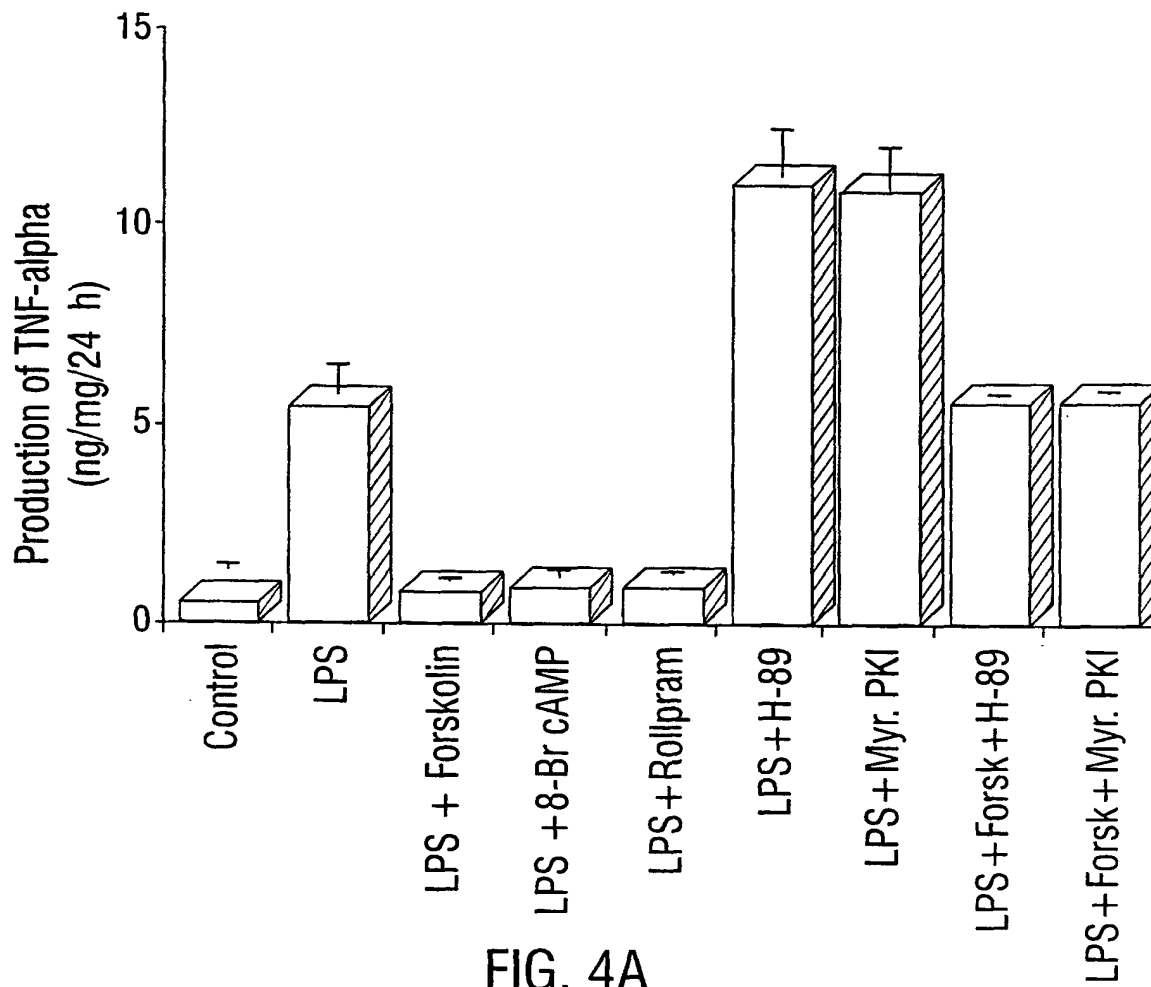


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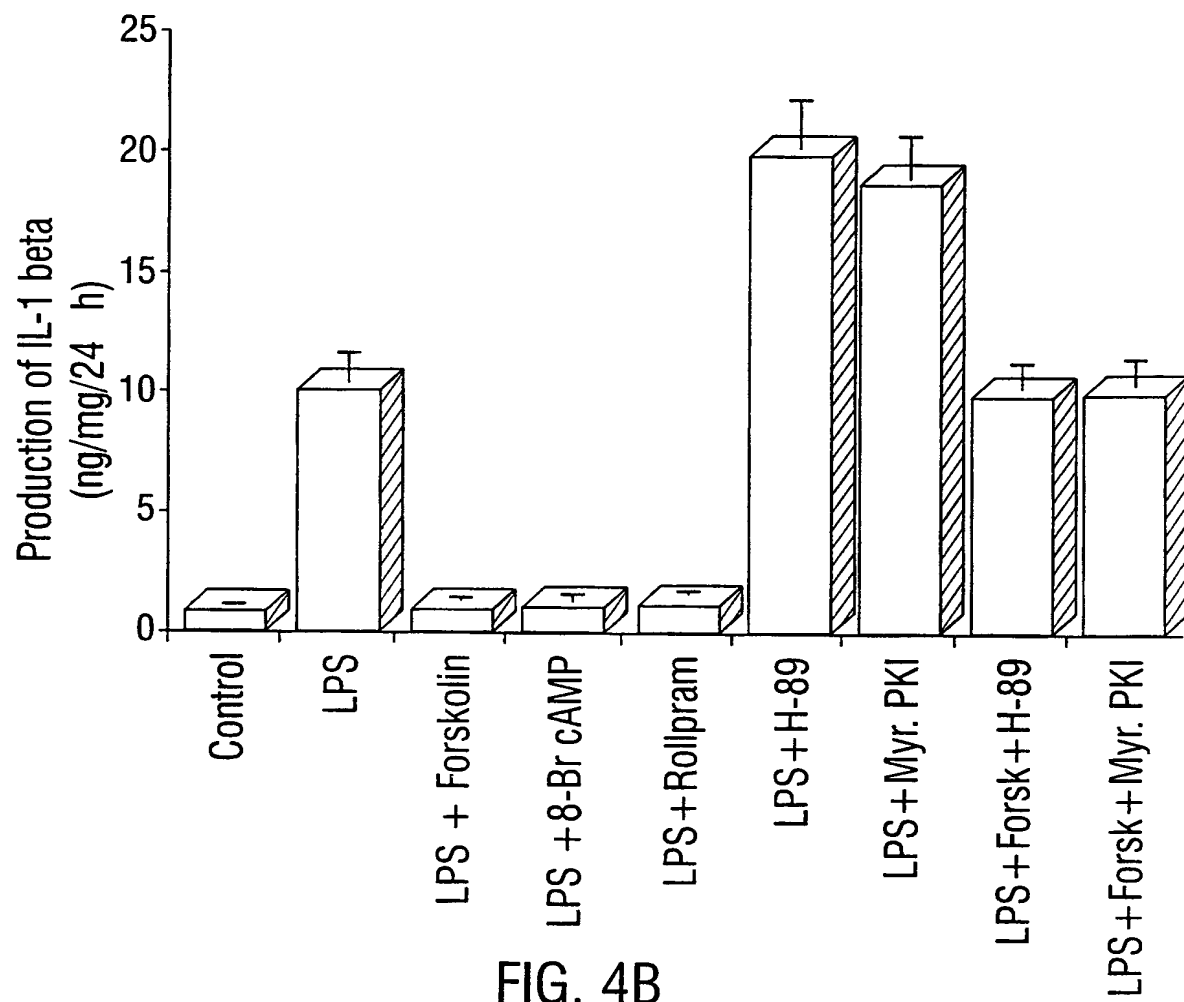
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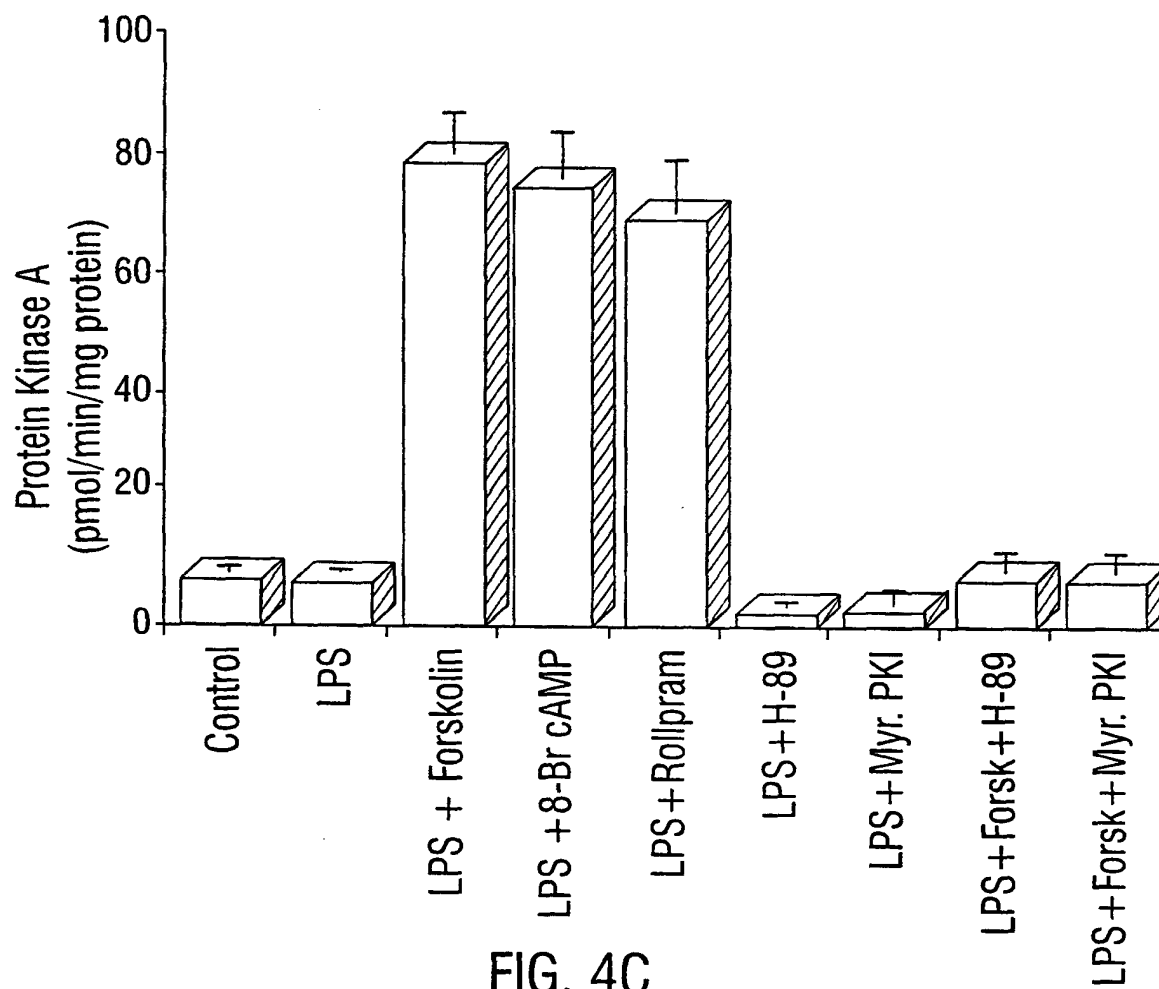
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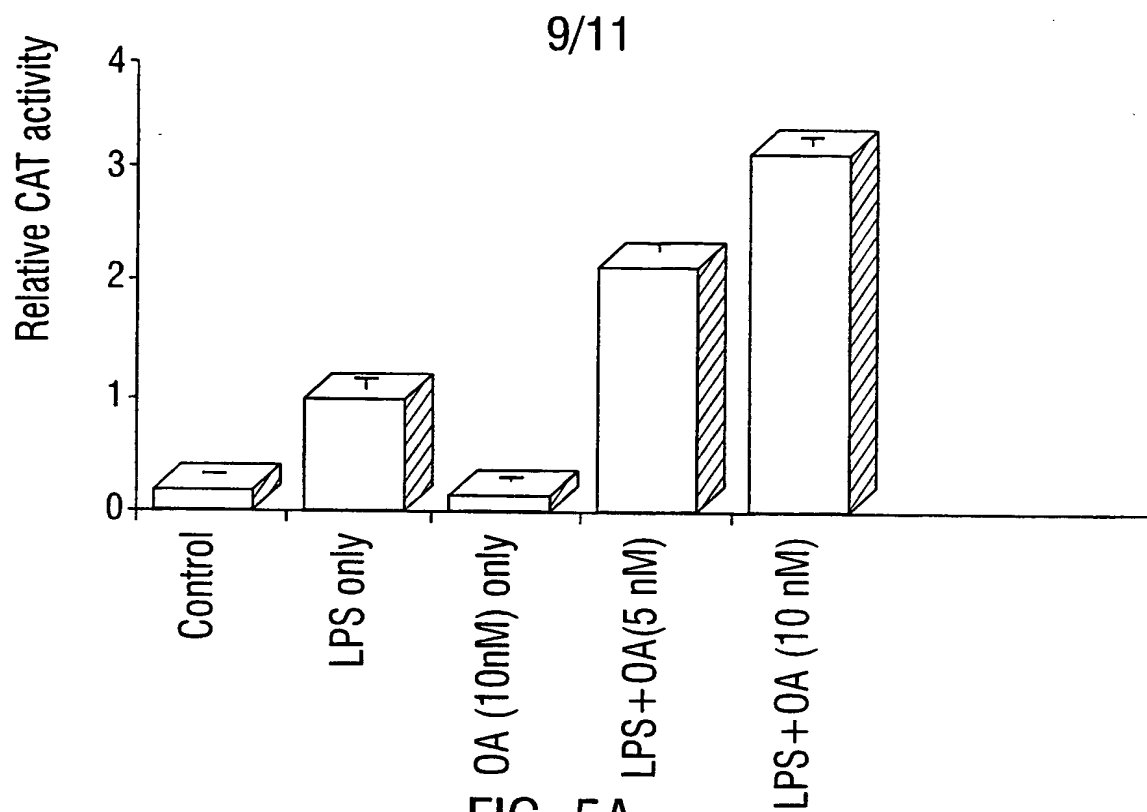


FIG. 5A

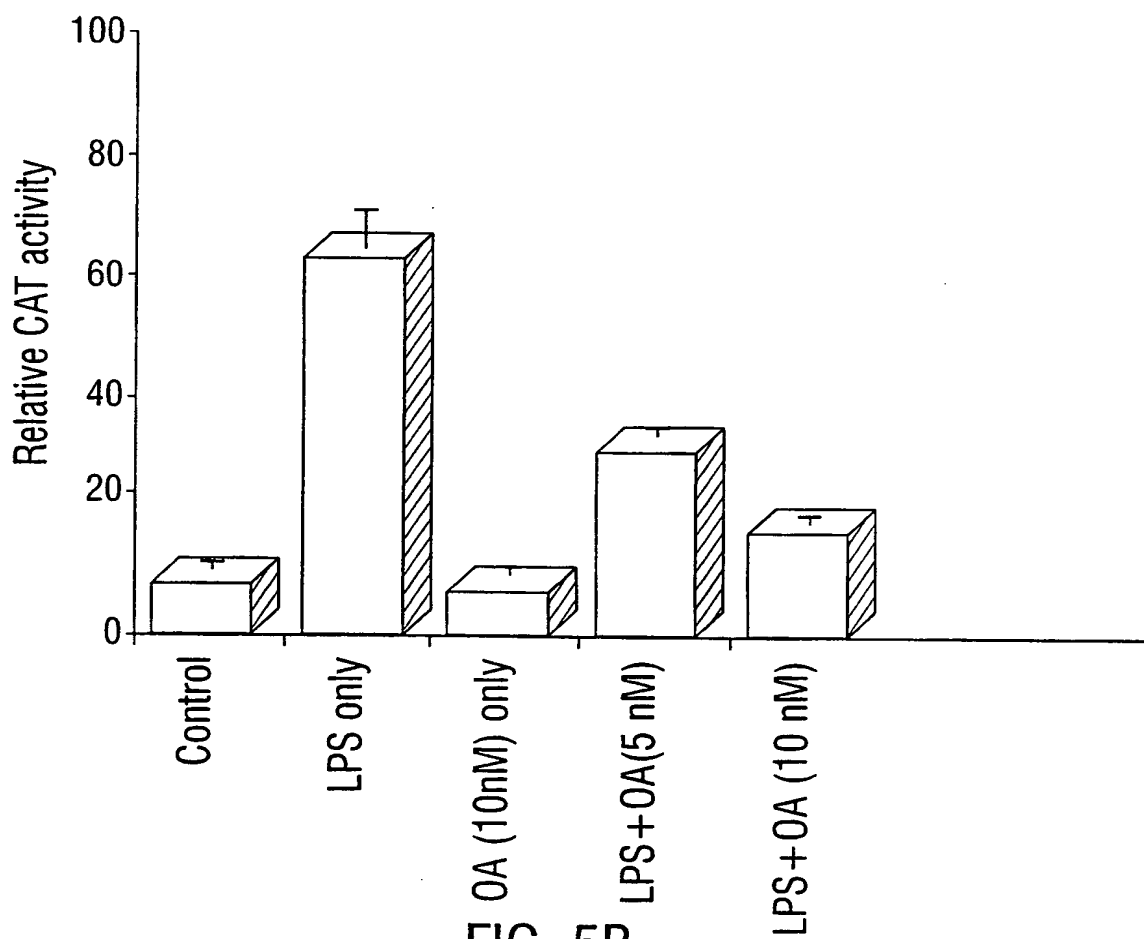


FIG. 5B

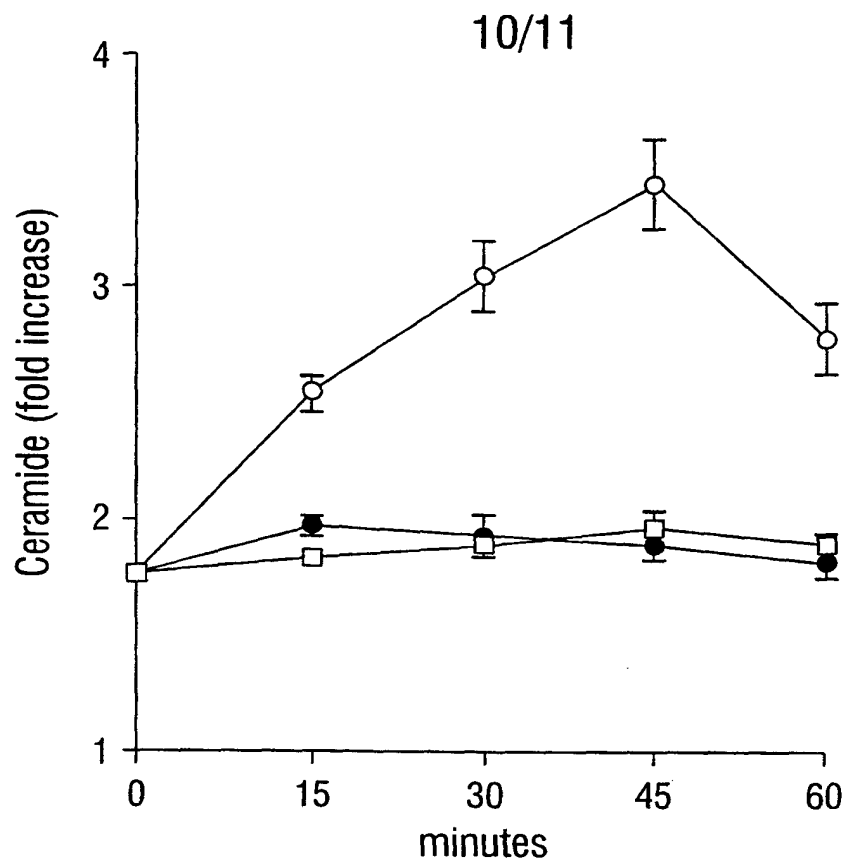


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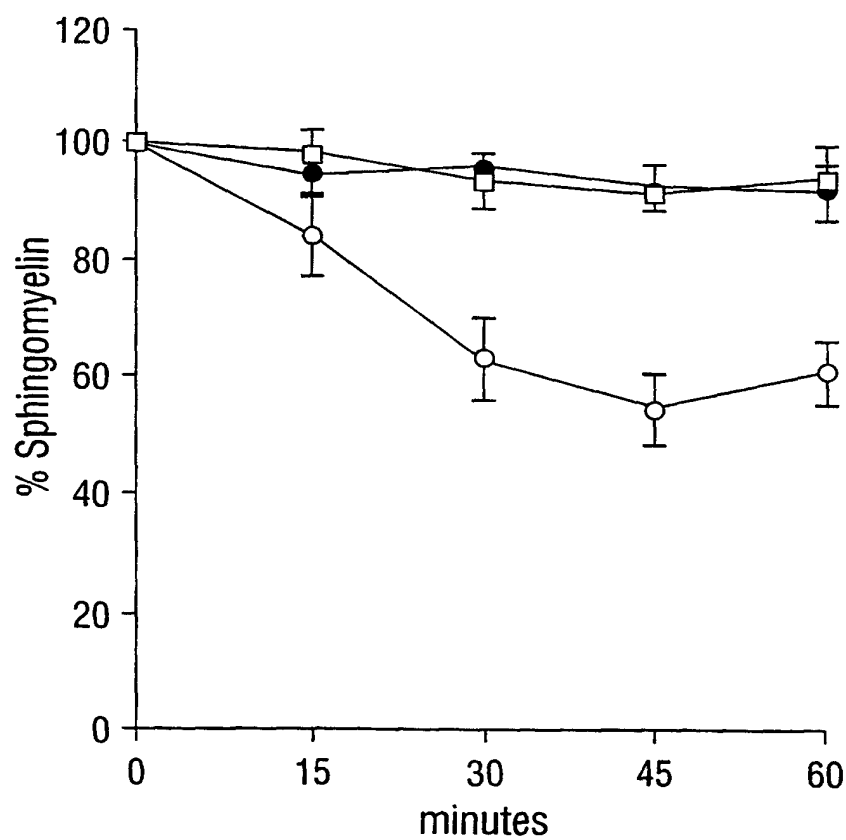


FIG. 6B

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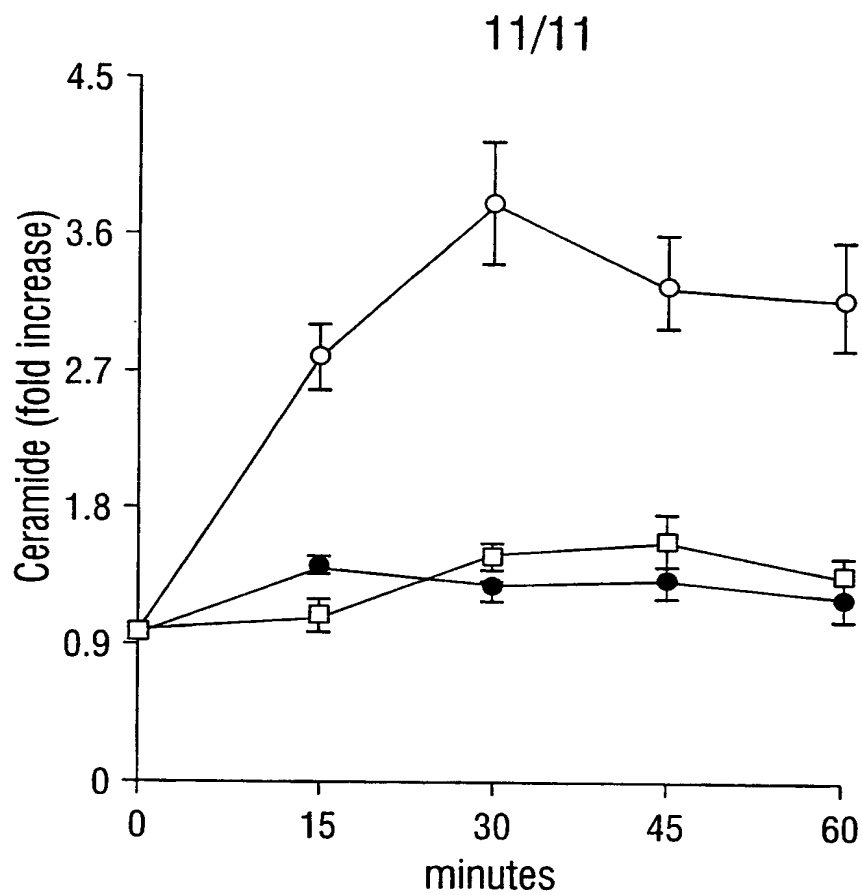


FIG. 7A

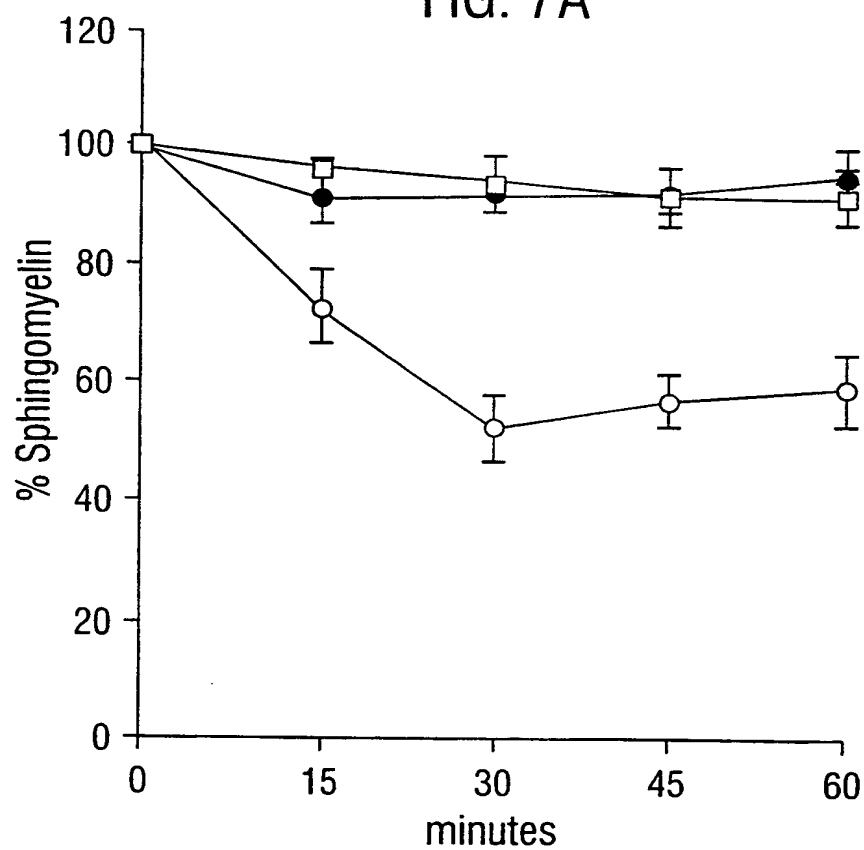


FIG. 7B

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/25360

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 45/00; C12N 9/99; C12Q 1/26, 1/34

US CL :424/278.1; 435/18, 25, 69.2, 184

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/278.1; 435/18, 25, 69.2, 184

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEM ABSTRACTS, DERWENT, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	US 5,695,761 A (DENHARDT et al.) 09 December 1997, column 3, lines 28-57.	1-75
P, A	WO 98/09653 A1 (SOCIETE DE CONSEILS DE RECHERCHES ET D'APPLICATION SCIENTIFIQUES) 12 March 1998.	1-75
A	US 5,545,625 A (GROSS et al.) 13 August 1996.	1-75
A	US 5,498,539 A (HARRISON et al.) 12 March 1996.	1-75

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 JANUARY 1999

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	PAHAN ET AL. Lovastatin and Phenylacetate Inhibit the Induction of Nitric Oxide Synthase and Cytokines in Rat Primary Astrocytes, Microglia, and Macrophages. Journal Clinical Investigation. December 1997. Volume 100, pages 2671-2679, see entire document.	1-75
Y	PAHAN ET AL. Increasing cAMP Attenuates Induction of Inducible Nitric-oxide Synthase in Rat Primary Astrocytes. 21 March 1997. Vol. 272. No. 12. pages 7788-7791, see entire document.	1-75

